EXAMINATION OF THE REPRODUCTIVE PERFORMANCE AND TISSUE-SPECIFIC METAL ACCUMULATION PROFILES IN FEMALE FATHEAD MINNOWS (*PIMEPHALES PROMELAS*) DURING CHRONIC WATERBORNE EXPOSURE TO INDIVIDUAL METALS AND BINARY METAL MIXTURES

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By

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Abstract

The overall objective of the research presented in this thesis was to evaluate the effect of waterborne metals, alone and in binary combination on the reproductive capacity of fathead minnows (FHM; *Pimephales promelas*) during chronic exposure. A wide array of reproductive endpoints across multiple levels of biological organization (molecular, biochemical, sub-organismal and population) was evaluated to understand the reproductive effects of individual metals and metal-mixtures in fish. In addition, the thesis also focused on determining the interactive effects of binary metal mixture exposure on tissue-specific (gill, liver, gonad and carcass) metal accumulation patterns in fish.

This thesis consists of two generalized methodological components - *in vivo* and *in vitro* experiments. The first component employed a 21-day FHM reproductive bioassay. Trios (1 male: 2 female) of FHMs were exposed to four different experimental treatments via water: control, two individual metals, and a binary metal-mixture. The individual metals used in this study were cadmium (Cd), copper (Cu), zinc (Zn) and nickel (Ni), alone and in binary combinations of Cd-Cu, Cd-Zn and Cu-Ni. In the reproductive bioassay, the effects of individual metals and binary metal-mixtures in female fish were assessed by evaluating serum estradiol level, hepatic mRNA expression of estrogen receptors (ER-α and ER-β), vitellogenin (Vtg) and metallothionein (MT), and ovarian histopathology, and a wide array of whole organismal reproductive endpoints including fish fecundity (cumulative egg production). The second methodological component (*in vitro* study) of my research was conceived based on the findings of first two reproductive bioassays (Cd-Cu and Cd-Zn), and focused on assessing the capacity of individual metals and metal-mixtures to directly affect estradiol production in FHM ovarian explants. The *in vitro* study also exhibited

some first definitive evidence indicating that metals can directly impair steroidogenesis, further corroborating the findings of the *in vivo* reproductive bioassays.

This thesis provided novel insights into the mechanisms of reproductive toxicity of individual metals, especially Zn and Ni. More importantly, my research demonstrated that metals in binary mixtures, irrespective of their apparent mode of toxic action, appear to act in a predominantly additive manner on fish fecundity, which was found to be the most sensitive reproductive endpoint in FHM bioassays. In general, the main findings of my thesis suggested that the interactions of metals elicit their reproductive effects in fish by altering estradiol production and/or hepatic vitellogenin synthesis, which possibly occur as an indirect consequence of metal-induced disruption of energy homeostasis in fish. The examination of tissue-specific metal accumulation revealed no notable interactions for any of the binary metal combinations examined, except an antagonistic effect of Cd on hepatic Cu burden, and Zn on hepatic Cd burden. However, none of these antagonistic interactions on tissue metal burden translated into amelioration of reproductive effects. On the other hand, the examination of ovarian histopathology revealed an additive effect of Cu and Ni, and Cd and Zn on follicular atresia. Collectively, these findings indicated that cumulative tissue burden of metals during chronic waterborne exposure to binary metal-mixtures contributes to the reproductive impairment in FHM by increasing the energetic cost of metal detoxification (increased metallothionein induction) and affecting the oocyte maturation process. Overall, this thesis demonstrated that waterborne exposure to metals in binary mixture impairs fish reproductive capacity essentially by disrupting the estrogen-mediated reproductive pathways.

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You gain strength, courage, and confidence by every experience in which you really stop to look fear in the face. You are able to say to yourself, 'I lived through this horror. I can take the next thing that comes along.' ~ Eleanor Roosevelt

Dedication

To my grandparents who were not able to see this thesis completed in person but were constant supporters of everything I did and always made sure I knew how deeply I was loved and were so very proud of me.

John R. Zimmer

Richard H. Driessnack

Lorraine E. Driessnack

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List of Abbreviations:

17- αOHP	17- α hydroxyprogesterone
α	Alpha
AAS	Atomic Absorption Spectroscopy
ANOVA	Analysis of Variance
AOP	Adverse outcome pathway
ATSDR	Agency for Toxic Substances and Disease Registry
β	Beta
Ca	Calcium
Cd	Cadmium
cDNA	Complementary deoxyribonucleic acid
Cl	Chlorine
Cr	Chromium
CTR1	Copper transporter
Cu	Copper
CYP19	cytochrome P450 aromatase
DMT	Divalent metal-ion transporter
DNA	Deoxyribonucleic acid
E2	17 β-estradiol/estrogen
EC_{50}	Effect concentration 50
ECaC	Mitochondrial rich chloride cell
EDC	Endocrine disrupting chemical
ELISA	Enzyme-linked immunoabsorbent assay
EnaC	Epithelial sodium channel
ER	Estrogen Receptor
FHM	Fathead Minnow
Fig	Figure
FSH	Follicle Stimulating Hormone
g	Gram
GnRH	Gonadotropin releasing hormone
GSI	Gonadosomatic Index
GtH	Gonadotropin
H	Hydrogen
Hg	Mercury
HPA	Hypothalamus-Pituitary-Adrenal
HPG	Hypothalamus-Pituitary-Gonad
HPI	Hypothalamus-Pituitary-Interrenal
HPT	Hypothalamus-Pituitary-Thyroid
Hr	Hour
IBMX	3-isobutyl-1-methylxanthene
IC50	Inhibition concentration 50
IHC	Immunohistochemistry
K	Potassium
K-S	Kolmogorov Smirnov
L	Liter

LC_{50}	Lethal concentration 50
LH	Luteinizing Hormone
LSI	Liversomatic Index
Mg	Magnesium
mg	Milligram
$MgCl_2$	Magnesium chloride
min	Minute
mL	Milliliter
mM	Millimolar
MT	Metallothionein
n	Sample Size
Na	Sodium
Ni	Nickel
ng	Nanogram
nM	Nanomolar
OECD	Organisation for Economic Co-operation and Development
P450arom	Cytochrome P450 aromatase
P450scc	Cytochrome P450 side chain cleavage
Pb	Lead
ppm	Parts per million
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Real-Time Polymerase Chain Reaction
StAR	Steroidogenic acute regulatory protein
T	Testosterone
TU	Toxic unit
µg	Microgram
µL	Microliter
µm	Micrometer
US EPA	United State Environmental Protection Agency
Vtg	Vitellogenin
ZIP	Zinc transporter
ZP	Zona pellucida
Zn	Zinc

NOTE TO READERS

This thesis is organized and formatted to follow the University of Saskatchewan College of Graduate Studies and Research guidelines for a manuscript-style thesis. Chapter 1 is a general introduction and literature review, including the project goal and objectives, and Chapter 6 contains a general discussion and conclusions tying the chapters together. Chapters 2, 3, 4 and 5 of this thesis are organized as manuscripts for publication in peer-reviewed scientific journals. Chapter 2 has been published in Comparative Anatomy and Physiology Part C, Chapter 3 has been published in Environmental Toxicology and Safety, and Chapter 5 has been submitted to Chemosphere. Full citations for the published research manuscripts are provided below. As a result of the manuscript-style format, there is some repetition of material in the introduction and materials and methods sections of the thesis. The tables, figures, supporting information and references cited in these research chapters have been reformatted here to a consistent thesis style. References cited in each chapter are combined and listed in the References section of the thesis. Supporting information associated with research chapters are presented in the Appendix section at the end of this thesis.

The content of Chapter 2 was reprinted (adapted) from Comparative Biochemistry and Physiology, Part C, (http://dx.doi.org/10.1016/j.cbpc.2015.10.009). M. Driessnack, A. Matthews, J. Raine, and S. Niyogi, "Interactive effects of chronic waterborne copper and cadmium exposure on tissue-specific metal accumulation and reproduction in fathead minnow (*Pimephales promelas*)" 179. 165-173. Copyright (2016) with permission from Elsevier.

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The content of Chapter 5 has been submitted to Chemosphere and is currently under revision. The manuscript was submitted under joint authorship with A. Jamwal and S. Niyogi

The author contributions to each research chapter included:

Dr. Som Niyogi (University of Saskatchewan) provided scientific guidance and input, manuscript review, comments and editorial corrections, as well as funding and direction for all the research.

Dr. Jason Raine (University of Saskatchewan) and Amber Matthews provided training in the theory and application of RT-PCR performed in Chapters 2, 3 and 5, as well as manuscript comments for Chapter 2. Dr. Jason Raine also provided input on the setup, running and maintenance of the proportional diluter system used to perform the reproductive bioassays of Chapters 2, 3 and 5.

Ankur Jamwal aided in the planning, set-up and final sampling of the reproductive bioassays performed in Chapter 2, 3 and 5. Assistance on the methodology and application of *in vitro* general practice and techniques employed in Chapter 4

CHAPTER 1:
GENERAL INTRODUCTION

1.0 Introduction

The overall goal of my doctoral research was to evaluate the effects of waterborne metals in binary mixtures on the reproductive performance and tissue-specific accumulation of metals in fathead minnow (FHM; *Pimephales promelas)*. A particular emphasis was placed on the reproductive endpoints across multiple response levels (molecular to whole organism) due to the close links between these endpoints and long-term population stability. It is crucial for us to understand the effects of metal mixtures on fish reproduction since such knowledge will help us in determining the potential long-term impacts of metal mixtures in aquatic ecosystems.

One of the most adverse consequences of industry is the release of contaminants into the environment, more specifically, into surrounding watersheds (Knapen et al. 2004, Cirillo et al. 2012). Exposure of aquatic organisms to multiple contaminants is a widespread issue due to both intentional and unintentional anthropogenic waste and by-product release (Weir et al. 2016). Whether exposure is the result of regulated effluent discharges, drainage from mining practices, spill events or run-off, the implications for aquatic organisms can be severe. The release of contaminants such as metals is an even more significant danger due to their persistence in the environment. Rates of metal elimination from contaminated environmental compartments can take months or years, and unlike organic compounds, metals and metalloids never truly degrade (Knapen et al. 2004, Meyer et al. 2005, Cirillo et al. 2012). Metals are ubiquitous in the environment and often exist naturally together. Many metals are also of vital importance to aquatic organisms because of their essential role in maintaining normal physiological functions. However,

serious concerns arise when environmental concentrations exceed a certain threshold above which adverse consequences can occur in organisms (Iwaski et al. 2010). Metals such as Cd, Cu, Zn, Pb, Hg, and Ni have become ubiquitous in the environment, thereby increasing the risk of causing detrimental effects in exposed aquatic organisms (Knapen et al. 2004). Field and laboratory-based studies have demonstrated that metal exposure can alter the vital molecular, biochemical and physiological processes, which are key to maintaining health, survival and ultimately reproduction in aquatic organisms including fish (Levesque et al. 2003, Rickwood et al. 2006, Weber et al. 2008, Werner et al. 2010).

To date, considerable progress has been made in setting metal regulations, standards and guidelines for the protection of aquatic life (Knapen et al. 2004). However, these parameters are almost exclusively based on single metal toxicity testing in simplified laboratory conditions (Meyer et al. 2005, Kamunde and MacPhil 2011). There are no doubts that single metal tests have provided valuable mechanistic knowledge regarding uptake pathways, potential modes of toxic action, and the establishment of cause and effect relationships. However, these simplified tests do not appropriately reflect actual mixture exposure conditions in the environment (Gauthier et al. 2006, Weir et al. 2016). The reality is organisms in exposed natural systems are almost always exposed to complex mixtures of sub-lethal levels of contaminants via the water and the diet (Dethloff et al. 1999, Pyle et al. 2001, Pyle et al. 2002, Gauthier et al. 2006, Rozon-Ramilo et al. 2011). Consequently, greater consideration should be placed in understanding metal mixture toxicity, as metals, when present in mixture, can modulate the uptake, distribution, and metabolism of each other. Interactions between metals may ultimately result in additive, synergistic, or antagonistic toxicity on the health, physiology and reproductive success of exposed organisms (Moreau et al. 1999, Norwood et al. 2003; Weir et al. 2016).

Despite the possibility that exposure to metal mixtures may affect organisms differently than exposures to individual metals, there are only a limited number of studies that have considered metal-metal interactions. Consideration of metal mixtures is of importance since such interactions may result in detrimental impacts at lower or higher concentrations than observed during single metal exposures (Kamunde and MacPhail 2011). Metal interactions in a complex mixture may be the result of competition for shared uptake sites on the biological surface, leading to increased bioavailability of certain metals while simultaneously decreasing the bioavailability of others through alterations in distribution, biotransformation, excretion or even speciation (Stewart 1999, Moreau et al. 1999, Pyle et al. 2002). With the knowledge that exposure to mixtures at chronic low doses are more applicable to real-world scenarios, it becomes evident that there is a need to investigate the effects of metal mixtures on biological endpoints in fish with a greater emphasis on reproduction, which is critical for long-term population stability. Population stability is of particular importance, as industrial and agricultural production continues to rise, so do the number of freshwater systems that may be impacted (Dethloff et al. 1999). It is particularly important to note here that Suter et al. (1987) evaluated various chronic response endpoints in several fish species (*e.g.,* FHM, rainbow trout, white sucker) and found that fecundity is generally the most sensitive effect.

1.2 Generalized fish reproduction

Optimal fish fitness and reproduction is achieved through the regulation of endocrine pathways such as that of the hypothalamus-pituitary-adrenal/interrenal axis (HPA/HPI), hypothalamus-pituitary-thyroid (HPT) axis and hypothalamus-pituitary-gonad (HPG) axis. The HPG axis is the primary axis responsible for the development and function of the gonads as well as reproductive behavior in teleosts (Ottinger et al. 2002, Norris and Lopez 2011). Disruption of

any of these axes may occur via the action of metals and chemicals mimicking natural hormones, blocking hormone action, interference with hormone synthesis, altering the secretion, availability, and metabolism of endogenous hormones or otherwise interfering with mechanisms of hormone action. These alterations can range from being transient and reversible to irreversible disruptions of reproduction, epigenetic impacts and localized population extinction (Norris and Lopez 2011, Scholz et al. 2013, Kerdivel et al. 2014).

1.2.1 Hypothalamus-pituitary-gonad axis in fish

Fish respond to social and environmental cues by sending signals to a range of brain centers via various chemical messengers and hormones culminating at the hypothalamus with the release of the decapeptide GnRH (gonadotropin releasing hormone) (Kah and Dufour 2011). The released GnRH then acts on the pituitary, through direct innervation in teleosts, stimulating the release of the gonadotropins (GtH); follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Arcand-Hoy and Benson 1998). The GtHs are then released into circulation and act on the gonads to influence gametogenesis and steroidogenesis. Sex steroids produced by the gonads are also released into the circulation, and they then bind to the hypothalamus and pituitary resulting in positive or negative feedback loops depending on the reproductive stage (Weltzein et al. 2004, Zohar et al. 2010).

1.2.2 Ovarian stages and histology

Ovaries in fish are typically a paired organ, attached to the wall of the body cavity. The structure and function of the ovary varies depending on the species' spawning strategy (*e.g.,* synchronous versus asynchronous) (Urbatzka et al. 2011). Synchronous spawning is demonstrated in species that typically only spawn once per year following a seasonal pattern, whereas

asynchronous spawning describes species that can continually spawn if environmental conditions favour such behavior.

Ovaries are comprised of germ cells, oogonia, and oocytes at various stages (Urbatzka et al. 2011). Oocyte development can be described as having five stages. During Stage I germ cells undergo primary oocyte growth stimulated by FSH. The primary oocyte becomes surrounded by follicle cells and begins forming the granulosa and theca cell layers. Stage II, the cortical alveoli growth stage is marked by the formation of the vitelline envelope and membrane-limited glycoprotein vesicles. During stage II of oocyte development, theca and granulosa cells continue to develop; these cells are integral to steroidogenesis. Transport of cholesterol via StAR (steroidogenic acute regulatory protein) into the inner mitochondrial membrane of theca cells is upregulated in response to GtHs (Nagahama 1994). Theca cells then convert the cholesterol into pregnenolone which is further converted by enzymatic pathways into testosterone (T). The resulting T is then released by the theca cells to the granulosa cells, which then convert T into estrogen by cytochrome P450 aromatase (Nagahama 1994, Norris and Lopez 2011). Estrogen as 17β-estradiol (E2) is released into the plasma where it binds to estrogen receptors (ER) in the liver to stimulate vitellogenin (Vtg) production and to modulate the hypothalamic and pituitary feedback loops. Stage III is the vitellogenic stage. Vitellogenin produced by the liver in response to plasma E2, secreted from the granulosa layer, is sequestered into the oocytes via endocytosis. The accumulated Vtg is then processed into yolk proteins, which will later be used by the developing embryo upon successful fertilization. In Stage, IV meiosis is reinitiated, and the oocyte matures in response to increasing LH levels and decreasing FSH. The final step, Stage V, is when mature eggs detach from the follicle cells and are ovulated (Arcand-Hoy and Benson 1998; Lubzens et al. 2010; Norris and Lopez 2011).

1.2.3 Role of estrogen receptors

Estrogen receptors are a class of ligand-dependent receptors and part of the nuclear receptor superfamily. This family of receptors also includes thyroid hormones, retinoic acids, steroids and vitamin D receptors (Vanacker et al. 1999, Cheung et al. 2013). There are two identified subtypes of ERs, ERα, and ERβ. For each of the subtypes, different isoforms have also been reported (e.g., ERα1) (Nagler et al. 2012). Estrogen receptors are rather promiscuous in nature meaning they will bind not just steroids but also metals, flavonoids, fatty acids and peptides (Norris and Lopez 2011). The roles of the different ER subtypes in fish is not entirely understood (Filby and Tyler 2005). ERα and ERβ have the highest level of expression in the liver, suggesting they play a role in E2 stimulated Vtg production (Filby and Tyler 2005). Nelson and Habibi (2013) recently hypothesized that in teleosts, ERβ might be more sensitive to E2 which in turn induces expression of ERα, and together they are responsible for stimulating Vtg production. Ultimately, the regulation of vitellogenesis and reproduction is a complex system where multiple estrogens and estrogen-related receptors (orphan nuclear receptors) play a variety of roles in reproductive success (Cheung et al. 2013).

1.2.4 Role of liver in vitellogenesis

The liver also plays a major role in fish reproduction in two primary capacities. First is through the production of Vtg, a phospholipoglycoprotein that provides vital yolk proteins and lipids, such as phosvitin and lipovitellin to the oocytes. Vitellogenesis is mediated by the action of E2 from the plasma entering hepatocytes and acting on nuclear ERs stimulating Vtg production. Vitellogenin is then transported back to the ovaries via plasma where it travels from the periphery of the ovarian follicles through multiple cellular and extracellular layers surrounding the oocytes, where it is finally internalized via selective cell-surface receptors (Mizuta et al. 2013).

The liver also acts in a second capacity as it plays a vital role in maintaining homeostasis through uptake, metabolism, storage, and redistribution of nutrients and other endogenous molecules along with metabolism and biotransformation of xenobiotics (Hinton et al. 2001, Hinton et al. 2008). Biotransformation of xenobiotics, as well as the production of detoxifying proteins such as metallothionein (MT), may alter hepatic function which can negatively affect resource allocation (Hinton et al. 2008). Stressed fish may then decrease or delay energetically expensive activities such as vitellogenesis and reproduction (Weber et al. 2008).

1.3 Study species of interest – fathead minnow

The FHM is a small-bodied fish and a member of the Cyprinidae, the largest family of freshwater fish (Jensen et al. 2001). Fathead minnows are of notable ecological importance in part due to their broad distribution across North American aquatic environments (Ankley et al. 2001, Ankley and Johnson 2004). Characteristics of mature adults include an average length of 50-75 mm and males weighing between 4 and 5 g and females between 2 to 3 g. Mature FHMs are sexually dimorphic with males being notably darker in color than females, and exhibit secondary sexual characteristics such as nuptial tubercles, a fleshy pad from nape to dorsal fin, commonly referred to as a fatpad, vertical body banding, and a caudal fin dot. Females tend to exhibit a pointed head and mouth profile, and they are lighter in colour and develop a fleshy ovipositor upon sexual maturity (Ankley et al. 2001, Ankley 2004, Parrot 2005).

Fathead minnows upon reaching sexual maturity demonstrate an asynchronous spawning behavior with spawning events occurring every 3-4 days under optimal conditions (temperature and light). A typical spawning event will consist of 50-150 eggs on average, though larger and smaller spawns do occur (Ankley et al. 2001, Jensen et al. 2001). Eggs are deposited by female FHMs to spawning substrates, which are then fertilized by the males. Successfully fertilized eggs

remain transparent for 36-48 hrs. Subsequently, the eggs progress to an eyed stage and typically hatch after a few days (Ankley et al. 2001). The well-known attributes of FHM reproduction and spawning behavior are a result of their extensive use in field and laboratory research, and in government and industry regulatory testing (Parrot 2005, Ankley and Villeneuve 2006, Rickwood et al. 2006, Villeneuve et al. 2011).

1.4 Metals

Research in my thesis focuses on four of the most commonly discharged metals (Cu, Cd, Ni, and Zn) that also demonstrate a tendency to be released in combination. More importantly, these metals are known to have common mechanisms of uptake, metabolism and/or toxicity in fish, and thus can interact with each other eliciting more than additive or less than additive effects in fish.

1.5 Copper

1.5.1 Copper in the aquatic environment

Copper (Cu) was one of the first metals to be used by humanity and is number 29 on the periodic table (Reilly 2006). Copper is present in the Earth's crust at about 50 mg/kg and can be found in surface waters at natural concentrations of 0.2-30 µg/L in freshwater (ATSDR 2004). However, anthropogenic activities can increase these levels of Cu to 100 µg/L and even as high as 200 µg/L in individual mine impacted watersheds (Grosell 2012). Activities that have been noted to contribute to increased Cu concentrations include base metal mining, electrical equipment, fossil fuel combustion, municipal wastewater discharge, manure, fertilizers, antifouling treatments such as paints and wood preservatives, fabricated metal production, and the leather industry. Copper may also enter the environment due to its frequent use as a fungicide and algaecide as means to control ectoparasites and bacterial infections in aquaculture (Newman and Unger 2003, Othaman

and Ahmad 2010). In 2009, it was estimated 15.8 million tons of Cu was produced from mining with a value of approximately $US 73 billion (Grosell 2012)

1.5.2 Physiology and toxicity of copper

Copper is known to be an essential trace metal for mammals and fish, serving as an integral component in enzymes and other important molecular complexes. However, Cu in excess can also be a potent toxicant (Watanabe et al. 1997, Bury et al. 2003, Reilley et al. 2006). One of the vital roles of Cu is facilitating cellular respiration via cytochrome c oxidase. Copper is also essential to many other critical enzyme functions including superoxide dismutase, lysyl oxidase, dopamine hydroxylase, tyrosinase, and ceruloplasmin (Watanabe et al. 1997, Bury et al. 2003). Organisms have evolved a range of mechanisms at the organismal and cellular level to maintain an optimal balance of Cu in the body. For example, during the synthesis of ceruloplasmin, the liver accumulates high concentrations of Cu. Subsequently this Cu-carrying protein is released into the blood to maintain a supply of Cu to other vital organs (Bury et al. 2003).

Fish are capable of accumulating Cu through waterborne and dietary exposure routes. Uptake from the water occurs at the gill epithelium, where Cu as Cu^{2+} competes with Na^{+} at uptake sites, but with an affinity that is four orders of magnitude greater than Na^{+}. Aqueous uptake of Cu occurs via the putative epithelial sodium channel (EnaC) and copper transporter (CTR1) (Bury et al. 2003). Uptake of Cu from dietary sources in fish has been suggested to occur mainly in the mid/posterior region of the intestines. Suggested entry pathways for uptake by enterocytes is through the divalent metal-ion transporter (DMT1) and the high-affinity Cu-transporter (CTR1), as well as through non-energy dependent, passive processes via Na^{+}-channels (Clearwater et al. 2002, Bury et al. 2003).

Acute toxicity from Cu typically occurs during waterborne exposure and mortality is often a result of iono-regulatory imbalance and acidosis that contributed to cardiovascular collapse. Copper essentially causes toxicity by disrupting Na^+ homeostasis in fish particularly by impairing branchial Na^+ uptake through the inhibition of Na^+/K^+-ATPase (Kamunde et al. 2005, Grosell 2012). Waterborne Cu exposure can also lead to increased plasma ammonia/ammonium levels possibly through elevated plasma cortisol stimulating protein catabolism. Also, Cu can contribute to respiratory acidosis by targeting carbonic anhydrase activity in the gills as well as by the impairment of swimming performance (Grosell 2012). In sublethal situations, toxicity is believed to be a result of increased reactive oxygen species (ROS) generation leading to oxidative stress. ROS have the potential to cause damage to DNA, proteins, and lipids within the organism if antioxidant mechanisms are overwhelmed. Oxidative stress can also be caused by Cu through inhibition of antioxidant enzymes and alterations in the mitochondrial electron-transfer chain (Moriwaski et al. 2008, Wood et al. 2012a). Waterborne Cu exposure is known to influence olfaction and mechano-reception in fish. Alterations to these sensory pathways can impair predator avoidance, prey localization, social interactions, homing and reproduction (Schlenk and Benson 2001, Grosell 2012).

Information on the reproductive effects of Cu in fish is very limited. Previous studies have found decreased reproductive output in brook trout exposed to 17 μg/L of waterborne Cu, and in bluntnose minnow at 18 μg/L of waterborne Cu, with water hardness at 200 mg/L (as $CaCO_3$) or greater (McKim and Benoit 1971, McKim and Benoit 1974, Horning and Neiheisel 1979). Another study that examined the effects of Cu on larval zebrafish found that 16 and 32 μg/L of waterborne Cu exposure, at a water hardness of 100 mg/L, affected survival and hatching (Dave and Xiu 1991). A recent study by Cazan and Klerks (2015) assessed the effects of waterborne Cu on the live

offspring bearing western mosquitofish. They reported that exposure to Cu did not affect the number of broods, but did decrease clutch size, and also increased the number of aborted broods, the number of dead offspring (Cazan and Klerks 2015).

1.6 Cadmium

1.6.1 Cadmium in the aquatic environment

Cadmium (Cd) is a naturally occurring metal, often associated with phosphate rock formations, and Zn, Pb, and Cu sulfide deposits. Natural processes such as weathering of minerals, forest fires, and volcanic emissions can lead to the natural releases of Cd. However, anthropogenic sources emit an estimated 10-fold greater amount compared to natural sources (Tilton et al. 2003). Cadmium is used in various applications including batteries, pigments, stabilizers, coatings and some alloys. Concentrations in water range from 1 to 400 µg/L Cd in compromised aquatic habits and a range of 1 – 10 µg/L Cd has been reported for drinking water (Newman and Unger 2003, Tilton et al. 2003, Wood et al. 2012b).

1.6.2 Physiology and toxicity of cadmium

Cadmium, unlike Cu and Zn, is a non-essential metal. Cadmium is a highly toxic and widely distributed pollutant. It has a low rate of excretion and a long biological half-life in organisms. It mainly accumulates in the kidney and liver as well as gonadal tissue in fish (Tilton et al. 2003, Henson and Chedrese 2004, Sellin and Kolok 2006, Kusch et al. 2007, Banni et al. 2011). Cd is often considered a classic Ca antagonist as it competes with Ca for uptake in the fish gill, and it is known to cause toxicity in fish by disrupting branchial Ca uptake and inducing hypocalcemia during acute exposure (Niyogi and Wood, 2004). It has also been noted that Cd can alter Ca homeostasis and hormone secretion from the gonads and pituitary by altering signal

transduction (Tilton et al. 2003). Fish are able to accumulate Cd through dietary and waterborne exposure pathways. Cadmium uptake in the gills is believed to occur apically via a lanthanum-sensitive voltage independent epithelium Ca^{2+} channel in mitochondria rich chloride cells (ECaC) and basolaterally via a high-affinity Ca^{2+}-ATPase and Na^{+}/Ca^{+} exchanger (McGeer et al. 2012). Uptake via the gut is not yet well characterized but is believed to occur at least in part via DMT1 and ZIP-8 transporters (Kwong et al. 2012).

Cadmium has been observed to impair endocrine function, through damage to gonadal tissue and impairment of gametogenesis and steroidogenesis, and reproductive output in fish (Sellin and Kolok 2006, Chouchene et al. 2011, Wang et al. 2014). A number of mammalian studies have demonstrated Cd-induced downregulation of pituitary hormones leading to impaired steroidogenesis, suppression of oocyte maturation and ovulation failure (Piasek et al. 2002, Lafuente et al. 2003). In fish, Vtg carries Ca and Zn from the liver to the ovaries. Cadmium previously accumulated in the liver can readily bind to Vtg, and the Cd-Vtg complex is efficiently taken up by the oocytes through receptor-meditated endocytosis. This transfer of Cd from the liver to oocytes has been demonstrated to result in increased rates of mortality, developmental deformities, and altered gonadal development in the offspring (Tilton et al. 2003, Meyer et al. 2005, McGeer et al. 2012, Wu et al. 2012). Cd has been shown to stimulate as well as inhibit gonadal steroid production in fish depending on dose and sex, possibly through altered Ca homeostasis, signal transduction disruptions and enzyme inhibition (Tilton et al. 2003, Lizardo-Daudt et al. 2007). Waterborne Cd exposure to the live-bearing western mosquitofish was found to decrease brood size and viability (Cazan and Klerks 2015). Overall, reproduction seems to be a sensitive endpoint for Cd exposure in fish.

1.7 Zinc

1.7.1 Zinc in the aquatic environment

Zinc (Zn) is the 23rd most abundant element in the Earth's crust. Zinc is typically found in ores that also contain Pb, Cu and Cd, and has been used by humans for over 2500 years in various applications (Reilly 2006, Wood et al. 2012b). The Zn concentrations in natural water vary in the range of 0.02 μg/L to 1000 μg/L, depending on the level of contamination (Hogstrand 2012).

1.7.2 Physiology and toxicity of zinc

Zn is an essential trace element and a component in more than 300 proteins functioning in growth, reproduction, vision and immune function and can also serve as an anti-oxidant (Bury et al. 2003, Clearwater et al. 2002, Banni et al. 2011). In fish, it is second only to iron (Fe) in the quantity used by the body in the metabolism of proteins, nucleic acids, carbohydrates, and lipids as well as by the immune system, neurotransmission and cell signaling (Bury et al. 2003, Hogstrand 2012).

Zn is assimilated through the gills and gut, and its status is tightly controlled due to the potential for toxicity at higher concentrations. Excess Zn is excreted via the bile, intestinal sloughing or the gills. Dietary uptake of Zn seems to be more tightly regulated than waterborne uptake (Hogstrand et al. 1996, Clearwater et al. 2002, Bury et al. 2003, Niyogi et al. 2007). Zinc uptake from the water occurs via the Ca^{2+} uptake pathways in the fish gills, which also leads to toxicity when excess uptake disrupts Ca homeostasis. Basolateral transfer of Zn in the gills may be associated with high-affinity Ca^{2+}-ATPases as they appear to be sensitive to waterborne Zn exposure (Niyogi et al. 2007, Hogstrand 2012). Calcium also inhibits Zn uptake, and it has been noted that waters with high hardness are protective of excess Zn exposure due to Ca competition. Dietary Zn uptake appears to occur primarily in the anterior intestine and is assumed to involve

the ZIP family of proteins (Bury et al. 2003). The general impression of Zn is that it is relatively non-toxic in the diet, as levels of 60-96 mg/kg did not result in effects on exposed rainbow trout (Clearwater et al. 2002, Bury et al. 2003). Additionally, the absorption and utilization of Zn in the diet is influenced by the chemical form of Zn as well as composition of the diet (Watanabe et al. 1997, Clearwater et al. 2002).

Again, much like Cu and Cd, there is limited data available on the reproductive effects of Zn exposure in fish. It has been suggested that Zn may affect reproduction by displacing Ca on Vtg (Hogstrand 2012). Brungs (1969) reported an 83% decrease in egg production in FHMs exposed to 180 μg/L Zn over a period of 10 months.

1.8 Nickel

1.8.1 Nickel in the aquatic environment

Nickel is the 22nd most abundant element and seventh most abundant of the trace metals often found in ores containing Cu. The world's largest Ni deposit is in Sudbury, Ontario, Canada which supplies around 25% of the world's Ni demand. Nickel is used in corrosion-resistant industrial equipment (specifically stainless steel), building materials, medical equipment, food and beverage containers, electroplating, jewelry and coinage, inks and dyes, strong magnets, electronic storage media and electromagnetic shielding (Reilly 2006, Pyle and Couture 2011). Nickel is commonly found in natural waters at concentrations of 1-10 μg/L, though heavily contaminated sites may reach concentrations as high as 1000 μg/L (Pane et al. 2003).

1.8.2 Physiology and toxicity of nickel

Nickel is a transition metal considered to be essential for terrestrial animals, as eight nickel-containing enzymes have been identified thus far. However, the essentiality of Ni and its modes of toxic action in fish are not very well understood (Brix et al. 2004, Pane et al. 2005, Pyle and

Couture 2011). Nickel is considered to be less toxic than Cd or Cu, and similar in toxicity to Zn across a range of fish species. The median lethal concentration for Ni in water has been suggested to be in the range of 4.5-9.8 mg/L (Pickering and Henderson 1966). It has also been reported that Ni is generally more toxic to early life stages compared to adults in fish (Pickering and Henderson 1966, Pyle and Couture 2011).

Nickel toxicity varies depending on the pH and hardness of the exposure water, with Ni being more toxic at higher pH/alkalinity, which is somewhat antithetical to the toxicity of other metals (Pyle et al. 2012). Acute exposure to waterborne Ni has been shown to interfere with Mg^{2+} reabsorption in fish kidney, which is assumed to be due to a sharing of transporters. Significant protein loss along with increased protease activity and free amino acid concentrations in gills and kidneys was found in fish exposed to acute levels of Ni (Sreedevi et al. 1992). Nickel has also been noted to induce morphological and histopathological damage in gill, liver, intestine and kidney tissue of fish (Pyle and Couture 2011).

Uptake of Ni at the gills is suspected to occur via low-capacity, high-affinity transporters at low exposure concentrations (Brix et al. 2004, Pyle and Couture 2011). In contrast, assimilation of Ni via the gut is assumed to occur mainly by low-affinity, high capacity transporters as wells as by passive diffusion (Leonard et al. 2009). In the blood stream, Ni can be transported as free Ni^{2+} ion, small ultra-filterable complexes, protein complexes or bound to blood cells. Nickel has been found to accumulate in multiple tissues in fish including gill, kidney, liver, brain, heart, muscle, bones, stomach, intestine and gonads during both waterborne and dietary exposures (Sreedevie et al. 1992, Canli and Kargin 1995, Ptashynski et al. 2001, 2002, Ptashynski and Klaverkamp 2002). In chronic reproductive studies, egg hatchability has been found to be a sensitive endpoint affected by waterborne Ni exposure (Dave and Xiu 1991, Pyle and Couture 2011). Pickering (1974)

reported that Ni decreases fecundity and egg hatching in fish at exposure levels greater than >380 µg/L.

1.9 Interactive effects of metal mixtures

1.9.1 Metal mixture general

Contaminants in mixtures whether natural, synthetic or a combination thereof, have the potential to cause antagonistic or synergistic adverse effects in organisms. However, at present mixtures remain one of toxicology's big unknown from the perspective of both human and environmental health (Mumtaz 2010). With respect to toxicity of metal mixtures, there have been an increasing number of studies examining the effects of metal mixtures on various aquatic organisms. However, most of these studies were conducted under acute exposure regimes, with a particular focus on how different metal combinations and dose ratios affect lethality and/or tissue metal accumulation patterns. A very limited number of studies have assessed the effects of low-dose metal mixtures on chronic endpoints including reproductive performance in fish. A review of the literature indicates that interest in metal mixtures and reproduction is not a new idea but is complex in its understanding. Some studies of interest in this multifaceted area of mixtures are highlighted in this section.

1.9.2 Toxicity of cadmium and zinc in mixture

Spehar et al. (1978) evaluated the effects of Cd (8.4 µg/L) and Zn (139 µg/L) alone and in combination on flagfish *(Jordanella floridae*) reproduction. They found that there was a decrease in male spawning behavior in single and mixed exposures compared to the controls. No differences were noted in adult growth or reproductive capacity except a decrease in mean spawning per female and embryo production in the mixture treatment compared to the control or single metal treatments. There were also no significant differences in embryo hatchability, survival and growth

in larvae from the exposed adults. The study also reported that a separate set of larvae raised from each of the treatments showed reduced survival when exposed to Cd and Zn singly, but not in the mixture. Whole body metal accumulation for adults and larvae demonstrated a significant increase in both Cd and Zn in the mixture treatment relative to the control, with no differences in either Cd or Zn between the mixture and respective single metal treatments.

1.9.3 Toxicity of cadmium and copper in mixture

The effects of combined Cd and Cu exposure in tilapia (*Oreochromis mossambicus*) was assessed by Pelgrom et al. (1994). They reported that whole body Cd content in fish exposed to Cd and Cu mixture was significantly decreased compared to the whole-body Cd content in fish exposed to Cd alone. It was also concluded that the kind of interaction (additive or synergistic) might in part be dependent upon the dose ratio of metals in the exposure. Those observations were further explored to examine the tissue-specific accumulation in tilapia during exposure to Cd and Cu mixture (Pelgrom et al. 1995). Results of this study indicated that the interactions of Cd and Cu were more pronounced at low doses. In the gill, intestine and gonad/ovary (females only), co-exposure of Cu and Cd resulted in a significantly greater Cd accumulation than the Cd-only exposure. Furthermore, co-exposure of Cd and Cu resulted in a significantly greater accumulation of Cu in the liver than that observed in Cu-only exposure.

A more recent study also evaluated the effects of Cd and Cu, singly and in mixture, on lipogenic metabolism in Javelin Goby (*Synechogobius hasta*) (Song et al. 2014). Fish were exposed to approximately 10% of 96hr LC_{50} values of each metal for 30 days. They reported that both Cd and Cu during individual exposures reduced fish growth, which was further reduced during exposure to the mixture relative to that in the Cu-only exposure. In addition, alterations in the activities of several key enzymes in the lipogenic pathway and hepatic histopathology were

recorded in both single metals and metal mixture exposures, resulting in a significantly greater hepatic lipid deposition in the mixture treatment.

The above studies highlight only a selected few studies in the literature pertaining to interactions of metals in binary mixture. Nonetheless they emphasize the complexities of assessing metal mixture toxicity in fish during chronic exposure. This also highlights the need to examine the chronic effects of metal mixtures on the physiology and reproduction in fish, also to elucidate the potential mechanisms underlying the interactive effects of metals. The latter aspect, in particular, has received very little attention to date.

1.9.4 Characterization of mixture toxicity

The interactive effects of metals in mixture can be determined to be either less than additive, additive, greater than additive or even antagonistic. These terminologies can be generally explained using a set of highly simplified hypothetical examples. For instance, Metal A and Metal B, alone and in mixture, were assessed for their interactive effects on fish fecundity. The easiest interaction to define is additivity, where Metal A reduces fecundity by 15%, Metal B by 25%, and the reduction is 40% in the mixture exposure, which would indicate a strict additivity (equal to the sum of individual metal effects). On the other hand, if the combined exposure resulted in a reduction of 60%, that would be a greater than additive effect (greater than the sum of individual metal effects), where as a reduction of 30% would be a less than additive effect (less than the sum of individual metal effects). It is to be noted though that the hypothetical examples used here are based on simple addition of percent response to determine interactions. However there are a variety of more complex approaches available for assessing mixture toxicity.

In this thesis, I used a derivation of simple additivity, as suggested by Norwood et al. (2003) and Niyogi et al. (2015), to determine the interactive effects of binary metal mixtures in *in vivo* fish reproductive bioassays (Chapter 2, 3 and 5). Considering the design of these studies where the mixture toxicity was assessed only at one fixed dose ratio, this was the most logical approach for assessing the interactive effects of metals on important chronic endpoints (e.g., fish fecundity). The simple additivity, which is based essentially on a response addition model, can be predicted by the following equation: Simple additivity = $[(x + y) - f] \times 100$

where x and y are the percentage fraction of a measured response induced by Metal A and B, respectively, and f is the interaction factor which is estimated by multiplying x and y. Mixture toxicity assessment by the response addition methods can also be characterized as independent action methods, as they assume that the toxic actions of chemicals or metals in mixture are statistically different from each other (Balistrieri and Mebane 2014).

In contrast, in my work examining the metal-mixture toxicity on *in vitro* ovarian steroid production (Chapter 4), a Toxic Unit (TU) approach was employed, which is considered to be a concentration addition model approach and assumes a common site of interaction (Norwood et al. 2003). In the concentration addition approach, metal mixture toxicity is assessed based on the conversion of the metal concentrations to an equi-toxic dose. This can be better described in terms of TU (Sprague 1970), where the concentration of each metal in the mixture is divided by the reference toxic dose (e.g., LC_{50} or IC_{50}) of an individual metal for any organism. This allows comparison of metals, that may have differing potency, at an equitoxic concentration (Norwood et al. 2003). Since my *in vitro* work allowed examination of toxic effects of specific combinations of binary metal mixtures at multiple dose ratios, it was appropriate to employ the TU approach to assess the interactive effects of metal mixtures.

1.10 Objectives

The research in my thesis was designed to develop our understanding of the chronic toxicity of waterborne metals (Cd, Cu, Zn, and Ni) in binary mixtures in a model freshwater fish (FHM), with especial emphasis on reproduction. The primary objectives of this research were three-fold: (i) to identify the combinations of these metals that elicit the most toxic response on reproductive performance, (ii) to identify sensitive reproductive and physiological endpoints that will help in chronic toxicity assessments of binary metal mixtures, and (iii) to elucidate the mechanistic underpinnings of the interactive effects of these metals in binary mixtures. I employed an integrative experimental approach and analyzed a wide array of chronic endpoints at various levels of biological organization (molecular to whole organism) to address my research objectives. In general, these endpoints include reproductive performance (e.g., egg production, spawning frequency, hatching success), biochemical and physiological assessments (e.g., circulating estradiol level, tissue-specific metal burden), and molecular responses (e.g., hepatic expression of genes that mediate estrogen responses, and metal detoxification). The specific objectives of this thesis were:

Objective 1: To determine and compare the tissue accumulation patterns of Cd, Cu, Zn, and Ni, individually and in binary mixture, during environmentally relevant chronic waterborne exposure in FHM.

Hypothesis: Metals with similar modes of branchial uptake (e.g., Cd and Zn) will demonstrate competitive interactions on the tissue-specific metal accumulation pattern. In contrast, metals that do not share common branchial uptake pathways (e.g., Cd and Cu, and Cu and Ni) will not exhibit any competitive interaction on their tissue-specific accumulation profile.

Objective 2: To evaluate the reproductive toxicity of Cd, Cu, Zn, and Ni, individually and in binary mixture, during environmentally relevant chronic waterborne exposure FHM.

Hypothesis: Metals in binary mixtures will elicit a greater degree of reproductive impairment compared to the effects of each metal individually. Metals with apparently similar modes of toxic action (e.g., Cd and Zn) will elicit strictly additive or less than additive reproductive toxicity, whereas metals with apparently disparate modes of toxic action (e.g., Cd and Cu, and Cu and Ni) will produce greater than additive effect on fish reproductive performance.

Objective 3: To characterize the physiological mechanisms by which the interactions of metals in binary mixture induce reproductive effects in FHM, using both in vivo and in vitro experimental approaches.

Hypothesis: Metals in binary mixture will impair fish reproduction by direct and/or indirect pathways. The direct pathway will involve impairment of estrogen synthesis/release as well as disruption of estrogen-mediated reproductive functions. Alternatively, the reproductive impairment may also occur as an indirect effect due to increased tissue metal burden and the associated increase in energetic cost of metal detoxification/sequestration.

As such, the thesis is organized in four data chapters (Chapters 2, 3, 4 and 5). Chapter 2 and 3 describe the studies that examined the *in vivo* chronic interactive effects of waterborne Cd and Cu, and Cd and Zn, respectively, in FHM. Chapter 4 was a follow-up study that evaluated how the interactive effects of Cd, Cu, and Zn on *in vitro* synthesis/release of estradiol in FHM ovary was influenced by the dose ratio of the metals in binary mixture. The final data chapter (Chapter 5) focuses on the *in vivo* chronic interactive effects of waterborne Cu and Ni in FHM.

CHAPTER 2:

INTERACTIVE EFFECTS OF CHRONIC WATERBORNE COPPER AND CADMIUM EXPOSURE ON TISSUE-SPECIFIC METAL ACCUMULATION AND REPRODUCTION IN FATHEAD MINNOW (*PIMEPHALES PROMELAS)*

2 Preface

This chapter focused on the interactive reproductive and physiological effects of Cu (a sodium-antagonist) and Cd (a calcium-antagonist) in fathead minnow (FHM), metals with apparently different modes of toxic action. Breeding trios of FHMs were exposed to waterborne Cd-only, Cu-only and a combined Cd-Cu for 21-days to assess changes in reproductive performance, tissue specific metal accumulation patterns and hepatic gene expression (estrogen receptors α and β, vitellogenin and metallothionein). Single metal responses indicated a significant reduction in reproductive output by Cu alone, while Cd alone was found not to affect the reproductive output. The results of the reproductive bioassay also indicated that the co-exposure of Cd and Cu led to a complete cessation of reproduction as well as produced a more than additive inhibition of hepatic vitellogenin gene expression, and induction of hepatic metallothionein gene expression in female fish. Overall, these findings suggested that the interactions of Cu and Cd impair fish reproduction by affecting estrogenic pathways as well as energy homeostasis.

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2.1. Introduction

Anthropogenic activities often lead to the contamination of aquatic ecosystems with metals, causing adverse consequences for aquatic life including fish. Our current knowledge on the toxicological implications of metal exposure in fish is primarily based on studies conducted

with single metal(s), even though in the real-world organisms inhabiting metal-contaminated natural environments are almost always exposed to metals in mixtures (Borgmann et al. 2008, Vijver et al. 2011). When present as mixtures, metals may interact with each other affecting uptake and metabolism, ultimately causing alleviation or augmentation of overall toxicological response (Daka and Hawkins 2006). A few recent studies have examined the toxic implications of metal mixtures on fish during short-term or acute exposures (Mebane et al. 2012; Balistrieri and Mebane 2014, Clemow and Wilkie 2015, Niyogi et al. 2015), however very little is known about the chronic effects of metal mixtures in fish.

Due to common natural and anthropogenic release processes, copper (Cu) and cadmium (Cd) often co-occur at elevated concentrations in contaminated natural waters (ATSDR 2004, UNEP 2010). Waterborne Cu is known to cause toxicity in fish mainly by disrupting sodium (Na^+) homeostasis during acute exposures (Grosell and Wood 2002, Morgan et al. 2004). Chronic or sub-lethal exposure to elevated concentrations of Cu results in Cu accumulation in target tissues (gill, liver, and kidney) in fish (Kamunde et al. 2005, Niyogi et al. 2006), in conjunction with a wide array of pathophysiological and toxicological effects. These effects include impaired growth and survival (Hansen et al. 2002a, Niyogi et al. 2006), disruption of ion (Na^+ and Cl^-) and Cu homeostasis (Niyogi et al. 2006), oxidative stress (Eyckmans et al. 2011), decreased metabolism and nitrogen excretion (De Boeck et al. 1995), reduced swim performance (Campbell et al. 2002), and compromised stress response (Gagnon et al. 2006). In addition, reproductive output in fish has also been suggested to be a sensitive and ecologically relevant endpoint for assessing chronic toxicity of Cu (McKim and Benoit 1971, Horning and Neiheisel 1979). The effect threshold for reproductive impairment in fish exposed to waterborne Cu has been reported to be 10-fold lower than the acute toxicity value (96 hr LC_{50}) of Cu (Horning and Neiheisel 1979). To date, the

mechanisms by which Cu impairs reproduction in fish remains unknown but could be an indirect effect of increased energetic cost of dealing with Cu-induced pathophysiological challenges.

Cadmium, on the other hand, is a calcium (Ca^{2+}) antagonist and is known to cause toxicity in fish primarily by disrupting Ca^{2+} homeostasis during acute exposures (Niyogi and Wood 2004). It is also reported to cause a wide range of adverse pathophysiological and toxicological effects during chronic exposure, including decreased growth and survival (Hansen et al. 2002b, Mebane et al. 2008), increased tissue-specific Cd accumulation (McGeer et al. 2000), and impaired ion (Na^+ and Ca^{2+}) homeostasis (McGeer et al. 2000, Reynders et al. 2006) and immune function (Zelikoff et al. 1995), oxidative damage (Cao et al. 2010), tissue and skeletal damage (Wangsongsak et al. 2007, Benaduce et al. 2008), and endocrine disruption (Foran et al. 2002, Lizardo-Daudt et al. 2008). Similar to Cu, Cd can also act as a reproductive toxicant, and previous studies have reported decreased spawning frequency and fecundity in fish chronically exposed to environmentally relevant waterborne Cd concentrations (Benoit et al. 1976, Sellin and Kolok 2006, Wang et al. 2014). Although the mechanisms underlying the reproductive toxicity of Cd remains to be fully understood, the reproductive effects are likely to be linked to impaired gametogenesis and/or steroidogenesis in fish. Cd has been found to impair gametogenesis in both male (decreased spermatids and spermatozoa) and female (decreased mature oocytes) fish during chronic waterborne exposure (Das 1988). In addition, Cd has been reported to reduce vitellogenesis and steroid levels, and delay oogenesis in female fish (Brown et al. 1994, Olsson et al. 1995, Foran et al. 2002, Tilton et al. 2003). Cadmium exposure has also been found to reduce mRNA expression of estrogen receptors in the liver and gonadotropin-releasing hormone in the brain of fish (Vetillard and Bailhache 2005).

Metals may interact with each other via shared routes of uptake and/or metabolic crosstalk, affecting their toxicokinetics and toxicodynamics. To date, the chronic interactive effects of Cu and Cd in fish have been investigated sporadically, and single and combined waterborne Cu and Cd exposures have demonstrated interactions between these two metals with respect to tissue metal(s) accumulation, and salt and water balance in the body (Pelgrom et al. 1994, 1995), and lipid metabolism (Song et al. 2014). In general, these studies have suggested that the interactions of Cu and Cd are complex and their combined effects cannot be predicted by the simple addition of effects of individual exposures of Cu and Cd. It is to be noted here that the interactive effects of chronic Cu and Cd exposure on fish reproduction have never been investigated previously. In this context, it is also important to note that generally reproductive output (fecundity) has been suggested to be the most sensitive endpoint (more sensitive than early life stage effects) in fish during chronic exposure to contaminants (Suter et al. 1987).

The present study was designed to examine the effects of waterborne Cd and Cu, both singly and in mixture, on reproductive performance, and also to gain insights into the mechanisms by which Cd and Cu cause reproductive toxicity in fish. We used a 21-day FHM reproductive bioassay, where fish were exposed to ~10% of 96 hr LC_{50} of each metal via water, individually or in combination, in addition to the control (no metals added). The main objectives of this study were: (i) to assess the interactive effects of waterborne Cd and Cu on the reproductive output of fish, (ii) to examine whether the interactions of Cd and Cu influence the accumulation of each metal in target tissues (gill, liver and gonad), and (iii) to evaluate how Cd and/or Cu exposure influence the hepatic expression of key genes that have important reproductive and metal detoxification functions (estrogen receptors (ER-α and ER-β), vitellogenin (Vtg), and metallothionein (MT)) and circulating estradiol level in female fish. We hypothesized that the

interactions of waterborne Cd and Cu would elicit greater than additive effect on the reproductive output of FHM.

2.2 Materials and methods

2.2.1. Experimental design and set-up

The current study was carried out in the Aquatic Toxicology Research Facility (ATRF) at the University of Saskatchewan. The experimental design and fish care procedures of this study were approved by the University of Saskatchewan Animal Care Committee, which followed the (FHM) aged approximately 12-months old from in-house cultures were used for the reproductive bioassay. Fish were maintained in flow-through 400-L tanks supplied with dechlorinated Saskatoon tap water (Ca^{2+} 44, Mg^{2+} 18, Na^{+} 26, K^{+} 3, Cl^{-1} 11, $SO4^{-2}$ 50, hardness 155, alkalinity 110 (both as $CaCO_3$), dissolved organic carbon (DOC) 2.5 (all in mg/L), pH 7.9). For water chemistry measurements, dechlorinated Saskatoon tap water was filtered through a 0.45-µm nylon syringe filters (Nalgene, NY, USA), and analyzed for cations and anions employing a Dionex ICS-3000 dual ion chromatography system (Dionex, CA, USA). DOC measurement was done using a Horiba total organic carbon (TOC) analyzer (Horiba, Kyoto, Japan). Water hardness and alkalinity were estimated using standard kits (LaMotte Company, MD, USA), and pH was measured using a pH meter (Mettler Toledo, ON, Canada). Fish were fed a commercial diet of frozen bloodworms (Sally's Bloodworms, San Francisco Bay Inc., CA, USA) at approximately 15% of bodyweight twice daily during both pre-exposure and exposure periods. Uneaten food and feces were siphoned out daily from the exposure tanks following 30 min of feeding period. Experimental design consisted of 4 treatments in a fully factorial two-way design: (i) control water (no added Cu or Cd), (ii) waterborne Cd only (6 µg/L, added as $CdCl_2$ (Sigma-Aldrich, MO, USA)), (iii) waterborne Cu only (60 µg/L, added as $CuCl_2$ (Sigma-Aldrich, MO, USA)), and (iv) waterborne

Cd and Cu in mixture (6 and 60 μg/L, respectively). The concentrations of Cd and Cu mentioned here are nominal concentrations and represented ~10% of 96 hr LC_{50} for Cd and Cu, respectively, for FHM under ambient water chemistry (unpublished data). The measured values for Cd and Cu exposure concentrations are reported in the results section (see below).

The FHM reproductive bioassay was carried out using the methodology as described by Driessnack et al. (2011) and Ouellet et al. (2013) and based on the assay standardized by Ankley et al. (2001). Each treatment was comprised of 5 replicate FHM trios (1 male: 2 female), with each trio individually housed in a 9-L glass aquarium, containing one breeding tile. Treatment water dilutions were made using a proportional dual head metering pump (Q2V, Fluid Metering Inc., NY, USA) drawing at a fixed rate from a control water head tank and the corresponding metal treatment head tank. Water from each tank was mixed within a pressurized manifold system (JCMSpecialties, SK, Canada), which delivered the appropriate treatment water to the corresponding aquaria. Pumps were set to a turnover of twice daily in each aquarium using a stroke rate controller (V300, Fluid Metering Inc., NY, USA). Tanks were maintained at 25 ± 2°C using a temperature-controlled water bath, and the light cycle was set to 16 hr:8 hr (light: dark).

2.2.1.1. Pre-exposure phase

The pre-exposure period was carried out to establish a baseline level of reproduction in FHM, which allowed for the selection of breeding trios to be used in the metal exposure. Fish were randomly selected from a stock culture, evaluated for fork length, bodyweight and secondary sex characteristics, and placed in individual aquarium (n = 40 trios). Egg production was assessed daily by examining the spawning tile for the presence of egg broods. Successful spawning events resulted in collection of eggs into a petri dish and then photographed for counting purposes. A micrometer was used for assessing egg and larvae photos taken during pre-exposure and exposure

phases. After 18 days, FHM breeding trios were selected for the exposure period based on 100% survival of adults, at least one spawning, and greater than 80% fertilization of eggs (OECD 2009). Statistical analysis of trios meeting baseline reproduction criteria was conducted at the end of the pre-exposure period to determine whether there were any significant differences in reproductive endpoints among trios to be used in the metal exposure. Normality was assessed using the Kolmogorov–Smirnov (K–S) test, and Levene's test was used to assess homogeneity of variance. If the assumptions were met, a one-way analysis of variance (ANOVA) was performed to analyze total eggs, and a Chi-Square test was also conducted for the number of spawning events. No significant differences ($p \leq 0.05$) were found.

2.2.1.2. Exposure phase

Upon completion of the pre-exposure phase, 20 FHM trios were selected randomly (5 replicates trios per treatment) and placed into exposure aquaria, and maintained for 21-days. Eggs were collected and counted daily as described above. Mean eggs per FHM trio in each treatment were calculated by dividing the total eggs produced over 21-days by the number of FHM trios used. For each brood, ten eggs were randomly selected for the determination of egg size using Image Pro Plus 6.1 (Media Cybernetics Inc., MD, USA). Collected eggs were also evaluated for fertilization success, hatching success, time to hatch, and 5-day larval survival and deformities. Eggs and larvae were maintained separately in appropriate exposure waters. Water samples (2-mL) were collected daily from an aquarium selected randomly from each treatment for the determination of total metal levels. At the end of the exposure phase, fish were anesthetized with MS-222, and fork length (mm), total body weight (g), and secondary sex characteristics (nuptial tubercles, body banding, and fin dot) were recorded. Blood samples were collected from females by caudal severance and allowed to clot on ice for the isolation of plasma, which was used later

for estradiol analysis. Gills, liver, gonads (testes and ovary) and carcass were dissected out from a female in each trio, rinsed for 20-s in deionized water, blotted dry, and placed in pre-weighed polythene tubes. The tubes were weighed again to determine the tissue weight to the nearest 0.01 mg. The liver tissue from the second female in each trio was collected for gene expression analysis in a pre-weighed vial containing approximately ten volumes of RNAlater (Life Technologies, ON, Canada).

2.2.2 Experimental analyses

Water quality in each treatment was monitored daily during both pre-exposure and exposure periods for temperature, dissolved oxygen (YSI meter, Yellow Springs Instruments, OH, USA), and ammonia (ammonia test kit, Rolf C. Hagen, AB, Canada). Water samples collected for metal analysis during the exposure phase were filtered through a syringe filter as described before, acidified (0.2% HNO_3, Trace metal grade, VWR, ON, Canada) and stored at 4 °C until analyzed. Fish tissue samples were digested in 5 volumes of 1 N HNO_3 at 60 °C for 48-h prior to their analysis for metal levels. Concentrations of Cd and Cu in the exposure water samples and fish tissue digests were analyzed by graphite furnace atomic absorption spectroscopy (AAnalyst 800, Perkin Elmer, CT, USA), with a practical detection limit of 0.2 μg/L for both metals. The quality control and assurance of metal analysis were maintained using appropriate method blanks and certified standards for Cd and Cu (Fisher Scientific, ON, Canada). In addition, the efficiency of Cd and Cu analysis in fish tissue samples were evaluated by analyzing a certified reference material (DOLT-3; National Research Council, Canada). The recovery of Cd and Cu in the reference material was 96% and 105%, respectively. For estradiol analysis, fish plasma samples were spun down at 2000 g for 10 min, and then analyzed using a commercial Estradiol ELISA Kit (Cayman Chemicals, MI, USA), following the instructions provided by the supplier.

For the evaluation of gene expression (Vtg, ER-α, ER-β and MT), whole female liver samples preserved in RNALater were removed and RNA isolated using an illustra RNAspin Mini Kit according to manufacturer's instructions (GE Healthcare, ON, Canada). The purified RNA was then quantified using a NanoDrop ND-1000 Spectrophotometer (Nanodrop Technologies, DE, USA), and samples were stored at −80 °C until cDNA synthesis could be performed. Synthesis of cDNA was carried out using a qScript cDNA Synthesis Kit (Quanta Biosciences, MD, USA) according to manufacturer's protocols to obtain a final sample concentration of 1 µg/µl cDNA for use in RT-qPCR analysis of gene expression. Synthesis of cDNA was carried out in a reaction volume of 20 µl comprised of 4 µl of qScript Reaction Mix (5X), qScript RT, and appropriate amounts of RNA and nuclease-free water, and then placed in the thermal cycler for: 1 cycle at 22 °C for 5 min, 1 cycle at 42 °C for 30 min and 1 cycle at 85 °C for 5 min and held at 4 °C until stored at −20 °C. The sequences for the target gene primers (ER-α, ER-β, Vtg, MT and β-actin (used as a house keeping gene)) used in the current study were taken from the literature where similar analyses in FHM were carried out (Werner et al., 2010). All of the primers were synthesized by Integrated DNA Technologies (IDT, IA, USA).

Prior to initiation of RT-PCR, standard curves were run for each pair of gene primer sequences to optimize linearity, detection range and efficiency. Standard curves were run using pooled control female liver cDNA samples (n= 3) in triplicate over a 10-fold serial dilution series. In addition, a no template sample (negative control) was run in triplicate for each primer sequence. RT-PCR analysis was carried out using an ABI 7300 Real-Time PCR System (Applied Biosystems, Life Technologies, ON, Canada) using a SYBR® Green PCR Master Mix (Applied Biosystems). Briefly, each cDNA sample was run in triplicate in a 96-well plate with a 25 µl reaction volume comprised of 12.5 µl of 2X SYBR® Green Master Mix, 1.5 µl of cDNA and

appropriate amounts of $MgCl_2$, reverse and forward primers for each target gene. The PCR conditions were set with an initial denaturation step of 10 min at 95 °C to activate AmpliTaq Gold®, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 60 s and finally a melt curve analysis. Relative expression of each target gene was normalized to the housekeeping gene (β-Actin), which was expressed at consistent levels in all experimental treatments. The resulting data were evaluated using the 2-ΔΔCT approach based on the method detailed by Schmittgen and Livak (2008). Results of this analysis were calculated relative to the control treatment, averaged and reported as changes in transcript abundance.

2.2.2.1 Statistical analyses

Data was analyzed using the statistical software package SPSS 22.0 (SPSS, Chicago, Il, USA). Normality of data was assessed using the Kolmogorov–Smirnov (K–S) test and homogeneity of variance was assessed using Levene's test. If the conditions of both normality and equal variance were met, a two-way Analysis of Variance (2-way ANOVA) was carried out. When assumptions were not met, data were log-transformed and re-assessed prior to analysis using a nonparametric test. If the assumptions were still not met, the Scheirer-Ray-Hare test, a non-parametric extension of the 2-way ANOVA, was performed. Parameters analyzed using these procedures included fish morphometrics and reproductive endpoints (except cumulative egg production), plasma estradiol levels, hepatic mRNA expression levels, and Cd and Cu concentrations in the exposure water and fish tissues. Cumulative egg production over 21-day exposure period was analyzed using the Kolmogorov–Smirnov (K–S) test. The differences in secondary sex characteristics among treatments were analyzed using the Chi- Square test.

2.3. Results

2.3.1. Exposure concentrations of metals

The measured concentrations of Cu and Cd in different treatments have been provided in Table 2.1. The concentrations of Cu and Cd in the exposure water were significantly elevated in respective single metal treatments relative to that in control. In the Cu and Cd mixture treatment, the exposure concentration of each metal was similar to that in their respective single metal treatments. General water quality parameters are presented in Table A1 of the Appendix.

2.3.2. Fish morphometrics and reproductive performance

No fish mortality occurred in any treatments during the exposure phase. There was no difference in fish weight (male and female), and secondary sexual characteristics among different experimental treatments (data not shown). Similarly, no differences in female hepatosomatic index (HSI) or gonadosomatic index (GSI) were recorded in the Cu and/or Cd treatments relative to the control (Table 2.2).

Cumulative egg production over 21 days of the exposure phase was found to be significantly reduced (~65%) in fish exposed to Cu alone relative to the control fish (Fig. 2.1). Cumulative egg production was not affected significantly by Cd only exposure. Interestingly, however, a complete cessation of spawning occurred in fish exposed to the Cu and Cd mixture (Fig. 2.1). A significant decrease in mean eggs/trio was also recorded in the Cu only exposure relative to the control and Cd only exposures, although spawning events/trio did not change among these three treatments (Table 2.2). In addition, egg size, and time to hatch were significantly lower in Cu only relative to both the control and Cd only exposures, while fertilization success was unaffected (Table 2.2). No differences in hatching success, 5-day larval survival or incidences of deformities were noted across the treatments (Data not shown).

2.3.3. Hepatic gene expression

Table 2.1: Measured concentrations of dissolved Cu and Cd in different treatment waters. Data are presented as mean ± SEM (n = 21). Significant differences in Cu concentration among different treatments are indicated by different letters. The asterisk (*) indicates a significant difference in Cd concentration relative to the control.

Treatment	Cu (µg/L)	Cd (µg/L)
Control	4.14 ± 0.6^{a}	ND
Cu Only	80.33 ± 6.8^{b}	ND
Cd Only	4.12 ± 0.5^{a}	5.56 ± 0.6*
Cu and Cd Mixture	74.01 ± 4.7^{b}	4.86 ± 0.5*

ND means not detectable (detection limit: 0.2 µg/L)

Table 2.2: Morphometrics and reproductive performance of fathead minnow (FHM) in different experimental treatments during the 21-day exposure period. Data are presented as mean ± SEM (n = 5 FHM trio). Different letters indicate statistical differences ($p \leq 0.05$) among treatments. Note that no spawning occurred in fish exposed to waterborne Cu and Cd mixture.

Endpoint/treatment	**Control**	**Cu only**	**Cd only**	**Cd and Cu mixture**
Female HSI	1.85 ± 0.31^{a}	2.49 ± 0.48^{a}	1.57 ± 0.38^{a}	1.84 ± 0.36^{a}
Female GSI	5.53 ± 2.67^{a}	6.40 ± 2.22^{a}	7.40 ± 1.15^{a}	7.85 ± 1.65^{a}
Mean Eggs/FHM trio	124.1 ± 19.4^{a}	38.3 ± 15.1^{b}	114.6 ± 27.4^{a}	-
Spawning events/ FHM trio	1.75 ± 0.5^{a}	1.0 ± 0.7^{a}	2.0 ± 0.8^{a}	-
Egg Size (mm)	1.37 ± 0.12^{a}	1.34 ± 0.16^{b}	1.40 ± 0.18^{a}	-
Time to hatch (days)	5.0 ± 1.5^{a}	4.5 ± 0.25^{b}	5.1 ± 1.8^{a}	-
Fertilization **Success (%)**	96.2 ± 2.1^{a}	97.1 ± 1.5^{a}	96.4 ± 2.2^{a}	-

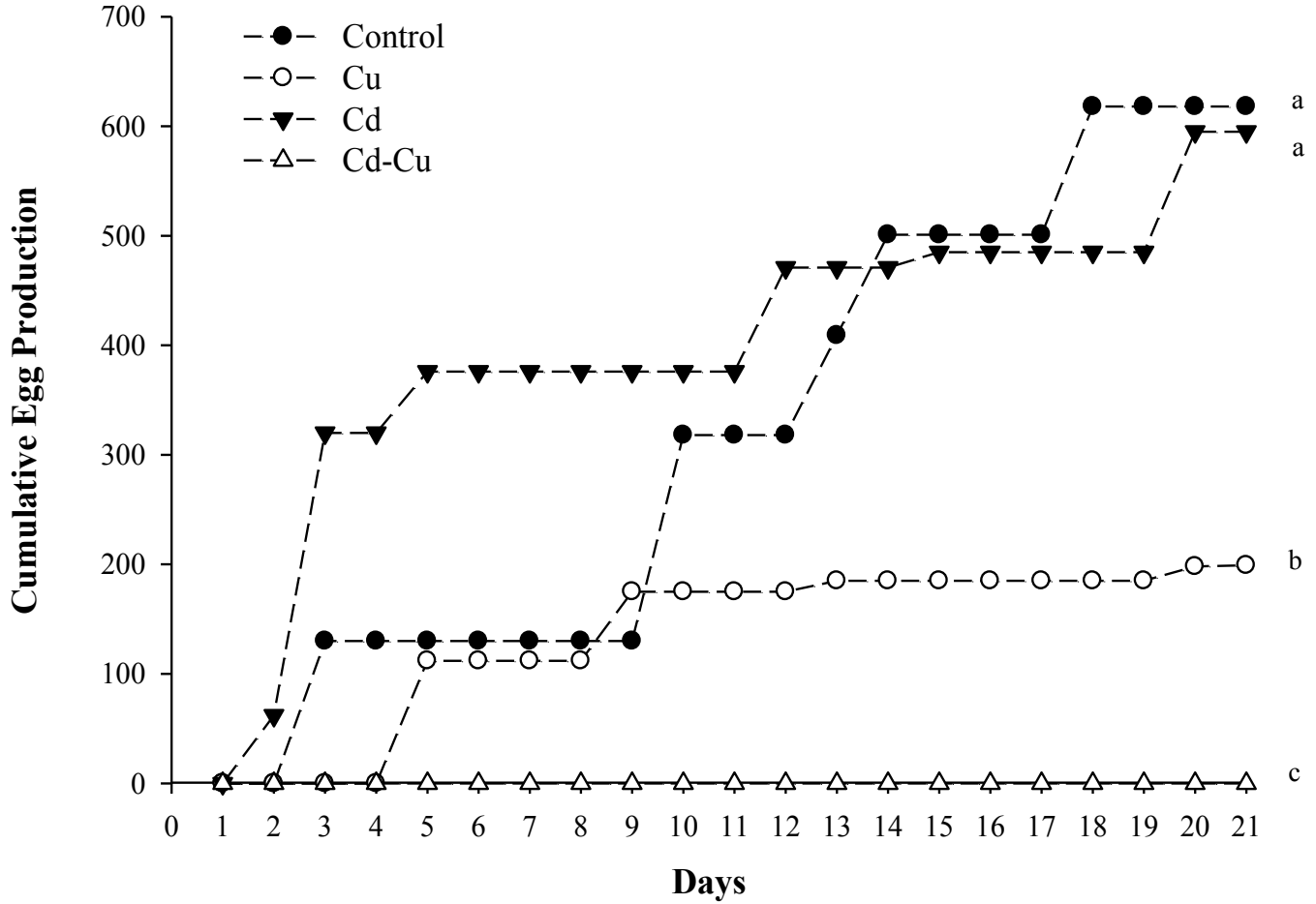

Figure 2.1: Cumulative egg production in fathead minnows over 21 days of exposure to waterborne Cu and Cd, individually and in mixture. Data were analyzed using Kolmogorov–Smirnov (K–S) test. Different letters denote significant differences among treatments, where $p \leq 0.008$ (Bonferroni Correction; $n = 5$ trios) are considered significant.

The livers of female FHM were analyzed to examine the expression of reproductively important genes (ER-α, ER-β, and Vtg) as well as the metallothionein (MT) gene. A small but significant decrease in abundance of ERα transcripts occurred in fish exposed to Cu only relative to the control. However the transcript abundance of this estrogen receptor was not influenced by Cd only exposure. Exposure to Cu and Cd mixture resulted in a marked increase (~15 fold) in abundance of hepatic ER-α transcripts in females (Fig. 2.2A). In contrast to ER-α, the abundance of hepatic ER-β transcripts was not affected by Cu only exposure, but increased significantly relative to the control during exposure to Cd alone as well as Cu and Cd mixture, with the maximal increase (~35 fold) in the latter treatment (Fig. 2.2B). The abundance of hepatic Vtg transcripts was also not affected by Cu only exposure, but reduced significantly (~3.5 fold) relative to the control following exposure to Cd alone. Exposure to Cu and Cd mixture, however, elicited a much greater reduction (~75 fold) in Vtg transcript abundance in the liver (Fig. 2.2C). Moreover, the transcript abundance of hepatic MT was not influenced by Cd only exposure, but increased marginally (~10 fold) relative to the control during exposure to Cu alone. Again, a significantly large increase (~200 fold) in hepatic MT transcript abundance was recorded in fish exposed to Cu and Cd mixture (Fig.2. 2D). In general, exposure to Cu and Cd mixture seemed to produce a greater than additive effect on the hepatic expression of all four genes examined, and a significant interaction of Cu and Cd was also recorded.

2.3.4. Tissue-specific metal accumulation

The accumulation of Cu and Cd was analyzed in the gill, liver, gonad, and carcass of female fish. No significant difference in the gill and ovary Cu concentrations was recorded among any of the experimental treatments (Figs. 2.3A and C). Cu burden increased significantly in the liver following exposure to Cu alone relative to the control. However no such increase of the liver Cu

accumulation was observed in fish exposed to Cu and Cd mixture, and a significant interaction of Cu and Cd was recorded (Fig. 2.3B). A significant increase in carcass Cu concentration was recorded in both Cu only, and Cu and Cd mixture treatments relative to the control (Fig. 2.3D). The exposure to Cd alone did not influence the Cu burden in any fish tissues.

In contrast, exposure to Cd alone, and Cu and Cd mixture resulted in increased Cd accumulation in all of the tissues examined compared to that in the control, and no interaction between Cu and Cd was found in any of the tissues (Figs. 2.4 A-D). Moreover, the tissue-specific Cd burden in fish exposed to Cu alone was similar to that in the control (Fig. 2.4).

2.3.5. Plasma estradiol

Plasma estradiol level in fish was not affected by either Cu only or Cd only exposure. However, exposure to Cu and Cd mixture resulted in a small but significant decrease in plasma estradiol concentration relative to that in the control, indicating a significant interaction between Cu and Cd in the exposure (Fig. 2.5).

2.4. Discussion

To the best of our knowledge, this is the first study to demonstrate that the interactions of chronic waterborne Cu and Cd exposure could cause greater than additive effects on fish reproduction. The exposure concentrations for Cu and Cd used in the present study are comparable to the levels often found in contaminated aquatic ecosystems (Thorton 1992, ATSDR 2004). In the present study, egg production was found to be the most sensitive reproductive endpoint during chronic exposure to metals. Our results revealed that cumulative egg production and mean eggs/trio decreased by 65% and 70%, respectively, in FHM exposed to waterborne Cu alone (~75 µg/L) compared to the control fish (Fig. 2.1; Table 2.2). Previous studies have reported reproductive impairment in fish during chronic waterborne exposure to Cu (McKim and Benoit

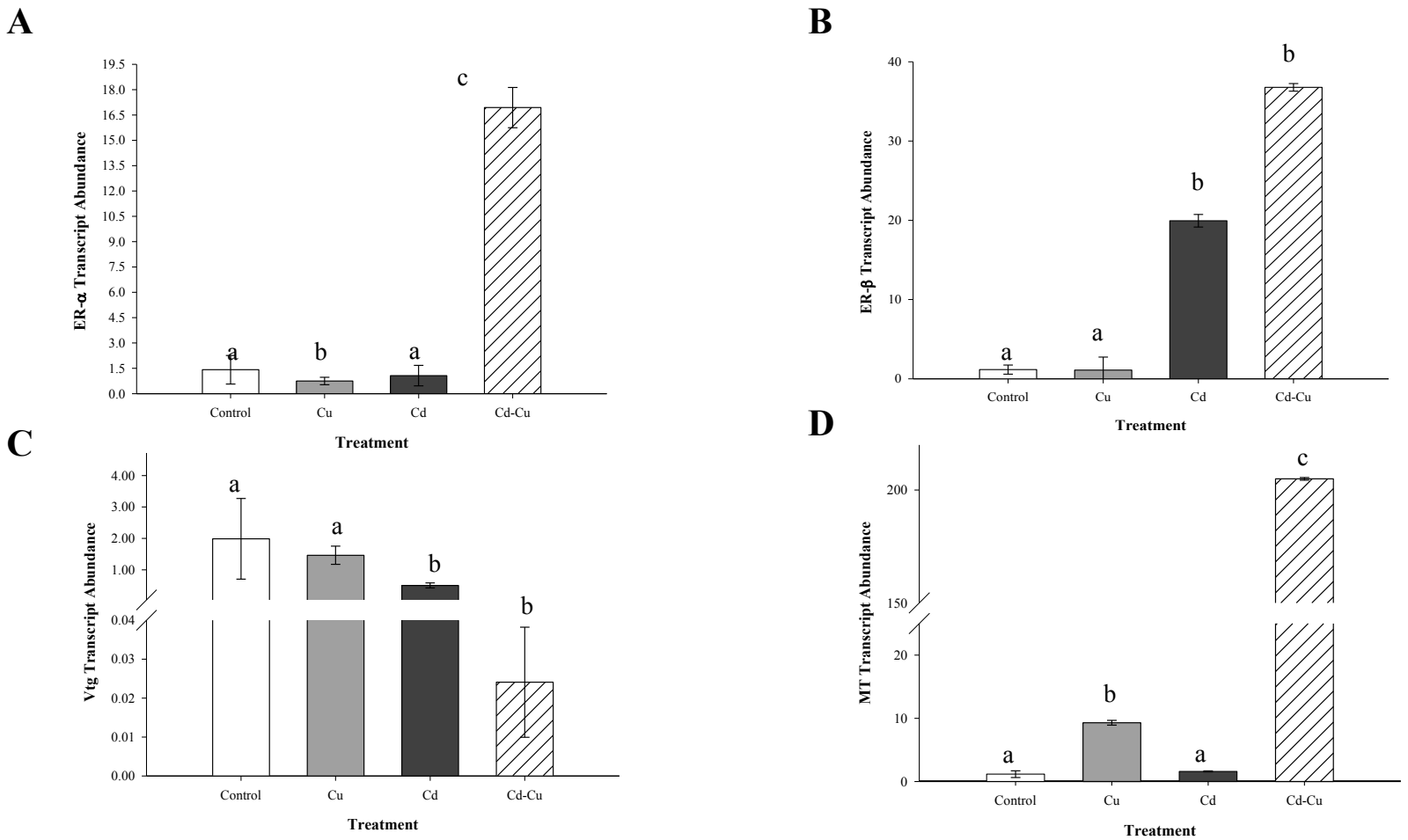

Figure 2.2: Hepatic expression of estrogen receptors (ER-α and ER-β), vitellogenin (Vtg), and metallothionein (MT) genes in female fathead minnows following 21 days of exposure to waterborne Cu and Cd, individually and in mixture. Transcript abundance of each target gene is expressed as relative to the housekeeping gene, β-actin. Data are shown as mean ± SEM(n=5). ER-β, Vtg, and MT expression data were analyzed by 2-way ANOVA, and ER-α data were analyzed by Scheirer–Ray–Hare test. Significant differences ($p \leq 0.05$) among treatments are indicated by different letters.

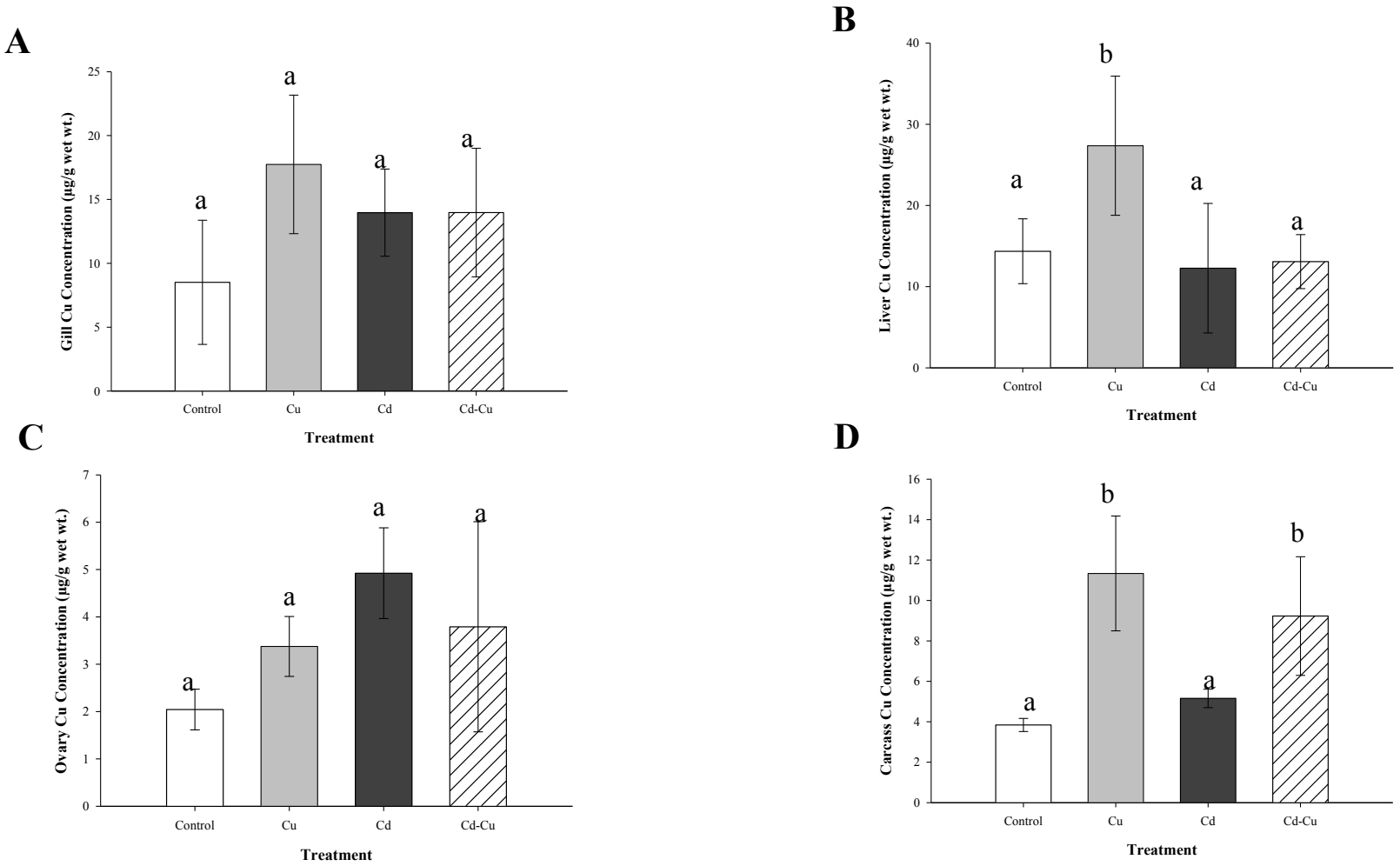

Figure 2.3: Tissue-specific concentrations of Cu in female fathead minnows following 21 days of exposure to waterborne Cu and Cd, individually and in mixture. Data are shown as mean ± SEM (n= 5). Gill, liver and carcass data were analyzed by 2-way ANOVA, and ovary data were analyzed by Scheirer–Ray–Hare test. Significant differences ($p \leq 0.05$) among treatments are indicated by different letters.

1971, Horning and Neiheisel 1979). Horning and Neiheisel (1979) examined the reproductive effects in bluntnose minnow (*Pimephales notatus*) exposed to a range of Cu concentrations in moderately hard water, comparable to the water used in the present study. They reported a decrease in egg production, spawning events and egg size at Cu concentrations ≥18 µg/L, with a complete cessation of spawning at 120 µg/L. Recently, Cazan and Klerks (2015) found that short-term maternal exposure to an environmentally relevant concentration of Cu (9.35 µg/L) decreased the brood size in the live-bearing western mosquitofish (*Gambusia affinis*). They also observed similar effects with short-term waterborne Cd exposure, although to a lesser degree.

In contrast to Cu, we found no significant effect of exposure to Cd alone (~5 µg/L) on any of the FHM reproductive parameters evaluated in the present study (Fig. 2.1; Table 2.2). Sellin and Kolok (2006) examined the concentration dependent reproductive effects of Cd in moderately hardwater using the same FHM reproductive bioassay used in the present study. They reported a significant decrease in egg production and spawning frequency, but only at the highest Cd concentration tested (50 µg/L). Wang et al. (2014) recently reported reduced egg production in FHM exposed to waterborne Cd (1–5 µg/L) under similar water chemistry conditions used in the present study. However, it is to be noted here that they did not find any effect of Cd based solely on egg production in the exposure phase, which is the recommended procedure for evaluating treatment effects on fecundity in the FHM reproductive bioassay (OECD 2009), and thus our findings are consistent with their study. Wang et al. (2014) noted the effects of Cd only when the fecundity of FHM pairs in the exposure phase was compared against the pre-exposure fecundity for both control and Cd-exposed groups. We also compared the FHM egg production between the pre-exposure and exposure phases for each treatment in the present study, which revealed a 28% decrease in the exposure phase following treatment with Cd alone (data not shown). However, this

decrease was not statistically significant when compared against the control group, which might have occurred due to the relatively lower replicate size in the present study (n = 5), in comparison to that (n = 8–14) used by Wang et al. (2014).

Interestingly, we observed a complete cessation of spawning in FHM exposed to the mixture of Cu and Cd (Fig. 2.1). Both Cd and Cu were present in the mixture in concentrations that were similar to their individual exposures. Thus, it can be suggested that Cu and Cd in mixture caused a greater than additive effect on the cumulative egg production in FHM, as their interactive effect was greater than the sum of individual effects of each metal. Jonker et al. (2004) examined the interactive effects of Cu and Cd in mixture on the reproduction of a nematode (*Caenorhabditis elegans*). Similar to our observation, they observed a greater than additive effect of Cu and Cd mixture, but only during the early life stages of *C. elegans*. They also suggested that the combined toxicity of Cu and Cd was influenced by their dose ratio in the mixture increased toxicity with more Cu and decreased toxicity with more Cd. Future studies should focus on investigating how the interactive effects of Cu and Cd in fish are influenced by their dose ratio in the mixture.

The effects of Cu and Cd mixture on reproductive output in FHM can occur via two possible pathways: (i) mixture directly interacting on the molecular/physiological mechanisms important for reproduction; and (ii) mixture affecting reproduction indirectly by inducing changes in energy allocation. The findings of the present study indicate that both the direct and indirect pathways might have played a role in inducing the effect of Cu and Cd on FHM reproductive output. We evaluated the hepatic expression of three genes (ER-α, ER-β, and Vtg) that play a critical role in regulating fish reproductive output. Vitellogenesis, which is a key process for the development and maturation of eggs in fish, is triggered by the binding of circulating estradiol to the estrogen receptors located in hepatocytes (Nelson and Habibi 2010).

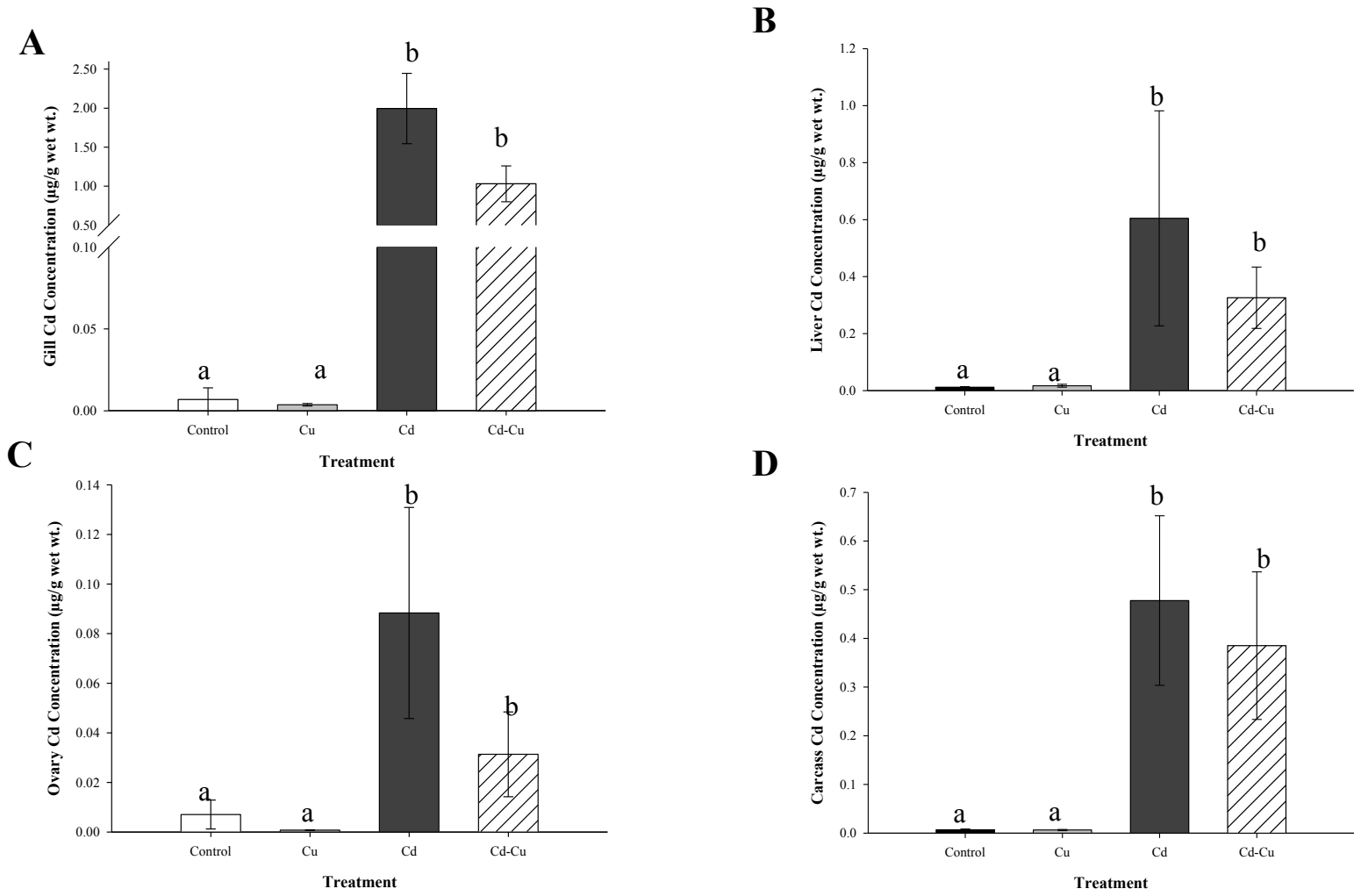

Figure 2.4: Tissue-specific concentrations of Cd in female fathead minnows following 21 days of exposure to waterborne Cu and Cd, individually and in mixture. Data are shown as mean ± SEM (n = 5). Gill and ovary data were analyzed by 2-way ANOVA, and liver and carcass data were analyzed by Scheirer–Ray–Hare test. Significant differences ($p \leq 0.05$) among treatments are indicated by different letters

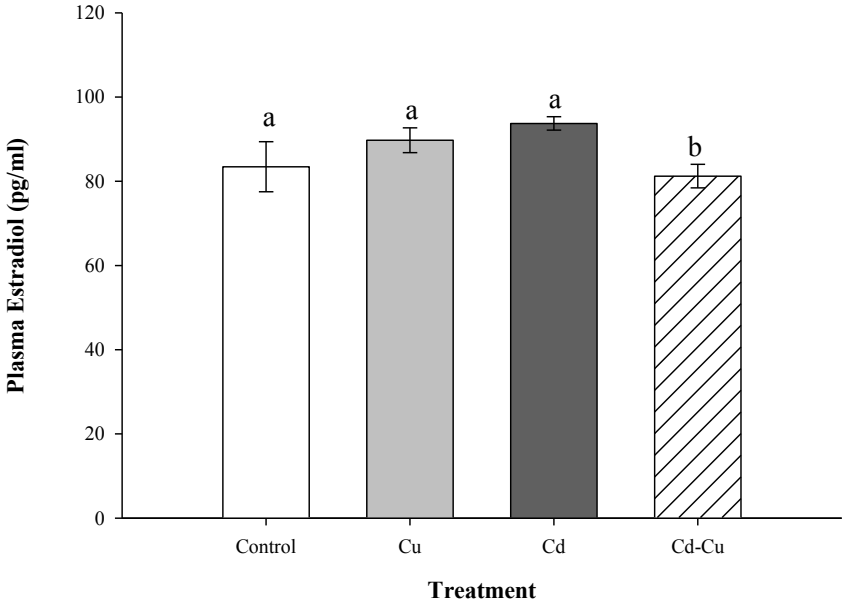

Figure 2.5: Plasma estradiol concentration in female fathead minnows following 21 days of exposure to waterborne Cu and Cd, individually and in mixture. Data are shown as mean ± SEM (n = 5). Data were log transformed and analyzed by 2-way ANOVA. Significant differences ($p \leq 0.05$) among treatments are indicated by different letters.

It has been suggested that estradiol first binds to ER-β, which induces vitellogenin synthesis and also increases the ER-α expression. Subsequently, with increased ER-α expression, hepatocytes are sensitized to further stimulation by estradiol, thereby producing more vitellogenin (Nelson and Habibi 2010). In mammalian systems, multiple metals including Cu and Cd have been found to be capable of binding to estrogen receptors and thereby disrupting the physiological functions regulated by estrogens (Dabre 2006). On the other hand, we also evaluated the hepatic expression of MT gene in FHM exposed to Cu and/or Cd. MTs are known to play a primary role in maintaining cellular homeostasis and detoxification of metals (Ahearn 2010). The sequestration of Cu and Cd occurs by displacement of Zn from their binding sites, leading to a release of free Zn that could induce de novo MT synthesis (Roesijadi 1996). Consequently, increasing accumulation of Cu and Cd could lead to the production of large amounts of MTs, which have been found to be negatively correlated with cellular energy allocation (Erk et al. 2008). Thus, increasing metabolic cost during metal exposure might also contribute to the adverse impact on the reproductive input of FHM.

Exposure to Cu alone did not influence the expression of FHM reproductive genes examined in this study, except a marginal downregulation of ER-α (Fig. 2.2A). Copper-induced downregulation of ER-α expression has been reported in human breast cancer cells, Mcf-7 (Martin et al. 2003). Nonetheless, no change in the expression of hepatic Vtg gene or the circulating estradiol level was recorded in FHM exposed to Cu. This suggests that the decrease in FHM reproductive output during Cu exposure occurred more likely due to the altered energy allocation to cope with Cu-induced physiological challenges, and less likely due to the direct endocrine effect of Cu. This argument is consistent with an almost 10-fold induction of hepatic MT gene expression

observed in FHM exposed to Cu alone. Altered energy allocation might have also led to the decrease in egg size observed in FHM exposed to Cu alone.

In contrast to Cu, exposure to Cd alone caused an upregulation of ER-β gene expression and a downregulation of Vtg gene expression in female FHM liver (Figs. 2.2B and C). Cadmium is known to be a potent metalloestrogen, and can activate estrogen receptors by binding to them with high affinity, blocking the binding of estradiol (Martin et al. 2003). The binding of Cd to estrogen receptors has been shown to diminish the transcriptional activity of estrogen receptors in rainbow trout (*Oncorhynchus mykiss*), ultimately affecting a number of estrogen mediated pathways including vitellogenesis (Le Guével et al. 2000, Vetillard and Bailhache 2005). No previous studies have examined the effect of Cd on hepatic expression of ER-β, whereas reduced expression of ER-α has been reported in rainbow trout during short-term exposure to Cd (5 μg/L) (Vetillard and Bailhache 2005). Recent mammalian evidence indicates that Cd exposure could also induce the expression of estrogen receptors (ER-α) (Ronchetti et al. 2013). It is possible that ER-β in FHM is more sensitive to Cd binding than ER-α, which resulted in its activation in the present study. Nonetheless, Cd-induced changes in the expression of ER-β and Vtg genes did not elicit any significant impairment of reproductive impairment. Moreover, although Cd is known to affect steroidogenesis directly (Henson and Chedrese 2004), we did not find any change in the circulating estradiol level in female FHM exposed to Cd alone, as reported previously by Wang et al. (2014), and Sellin and Kolok (2006) as well. In addition, no change in the hepatic expression of the MT gene was observed during exposure to Cd alone (Fig. 2.2D), although it resulted in a significant increase in tissue-specific Cd accumulation (Fig. 2.4). This indicates that the endocrinal and/or pathophysiological changes induced by the Cd exposure alone did not reach a threshold that culminates in the significant impairment of reproductive output in FHM.

Interestingly, however, exposure to Cu and Cd mixture resulted in a significant increase in the hepatic expression of both estrogen receptors (ER-α and ER-β) genes compared to the control or single metal treatments (Figs. 2.2A and B). The increased expression of ER-α and ER-β occurred probably due to the combined metalloestrogenic activities of Cu and Cd (Darbre 2006). Cu and Cd co-exposure also induced a marked decrease in the hepatic expression of Vtg gene, indicating a major disruption of vitellogenesis, which might have been an important factor that led to the cessation of spawning. It is also important to note that plasma estradiol level was significantly reduced in female FHM exposed to Cu and Cd mixture (Fig. 2.5), which might have occurred due to the crosstalk of Cu and Cd with the steroidogenesis pathway in fish (Handy 2003, Lafuente 2013). Moreover, co-exposure to Cu and Cd also produced a sharp upregulation of hepatic MT gene (Fig. 2.2D), indicating an increased metabolic cost of coping with elevated Cu and Cd body burden. Thus, it is possible that altered energy allocation during exposure to Cu and Cd mixture might have contributed, at least in part, to the downregulation of steroidogenesis and vitellogenesis, eventually affecting fish spawning.

In the present study, exposure to waterborne Cu and Cd, alone or in combination, significantly increased tissue-specific accumulation of respective metals in FHM (Figs. 2.3 and 2.4). No interaction of Cu and Cd accumulation was recorded in any tissues in fish exposed to Cu and Cd mixture, with the exception of the liver where Cu burden was reduced in the presence of Cd. Cadmium accumulation increased in all of the tissue types (gill, liver, ovary, and carcass) examined in fish exposed to Cd alone. Wang et al. (2014) previously reported a similar increase in tissue-specific Cd accumulation in FHM exposed to the same levels of waterborne Cd as used in our study. In contrast to Cd, an increased Cu accumulation was observed only in the liver and carcass during exposure to Cu alone. Liver has been suggested to be the primary site of Cu

metabolism and accumulation in fish (Kamunde et al. 2003). In general, the apparent lack of interaction between waterborne Cu and Cd accumulation could be due to the differences in their uptake pathways in fish. Waterborne Cd uptake occurs mainly via the branchial calcium uptake pathway (Niyogi and Wood 2004), whereas waterborne Cu is predominantly taken up via the branchial sodium uptake pathway (Grosell and Wood 2002). In contrast to our study, Pelgrom et al. (1994) reported a decrease in whole-body Cd burden in fish chronically exposed to waterborne Cu and Cd mixture relative to that in fish exposed to waterborne Cd alone, and no interactive effect of Cu and Cd on whole-body Cu burden. Recently, Niyogi et al. (2015) demonstrated that waterborne Cu inhibits short-term acute uptake of Cd in fish and vice versa, suggesting their interactions through shared uptake pathways that are yet to be characterized. Clearly, the interactive effects of Cu and Cd are complex, and further studies are required to understand how this interaction is influenced by different variables such as dose ratio of Cu and Cd in the mixture, and exposure duration and routes.

2.5. Conclusion

Overall, our study has revealed that chronic waterborne exposure to Cu and Cd mixture can cause a greater than additive effect on the reproductive output in FHM. Fish fecundity was found to be the most sensitive endpoint for assessing the reproductive effect of Cu and Cd mixture. The evaluation of circulating estradiol levels, and hepatic expression of estrogen receptors (ER-α and ER-β), Vtg and MT genes in female fish suggested that the greater than additive effect of Cu and Cd on the reproductive output was likely mediated by the disruption of estrogen regulated vitellogenesis, and energy homeostasis. Moreover, our study also indicated that chronic exposure to waterborne Cu and Cd increases the tissue burden of both metals, which can also be attributed to the toxicity of Cu and Cd mixture.

CHAPTER 3:

INTERACTIONS OF WATERBORNE CADMIUM AND ZINC DURING CHRONIC EXPOSURE ON TISSUE-SPECIFIC METAL ACCUMULATION AND REPRODUCTION IN FATHEAD MINNOW (*PIMEPHALES PROMELAS*)

3 Preface

This chapter focused on the interactive reproductive and physiological effects of cadmium and zinc in fathead minnow (FHM) - metals with an apparently similar mode of toxic action (calcium antagonists). Mature FHM breeding trios were exposed to waterborne Cd-only, Zn-only and a combined Cd-Zn for 21-days to assess changes in reproductive performance, tissue specific metal accumulation patterns and hepatic gene expression (estrogen receptors α and β, vitellogenin and metallothionein). Single metal responses indicated a significant effect of Zn-only exposure on reproductive performance, while Cd alone was found not to affect reproductive performance in FHM. The results of the reproductive bioassay also indicated that the co-exposure of Cd and Zn caused an additive reduction of reproductive output. Zinc was found to decrease the accumulation of Cd in the liver, and increase hepatic MT expression during co-exposure. Co-exposure of Cd and Zn also impaired hepatic Vtg expression and increased the frequency of follicular atresia in ovarian tissue. Overall, these findings suggested that Zn and Cu in binary mixture induce reproductive toxicity mainly by disrupting estrogen-mediated reproductive functions in fish.

Driessnack M.K., Jamwal, A., Niyogi, S. 2017. Interactions of waterborne cadmium and zinc during chronic exposure on tissue-specific meal accumulation and reproduction in fathead minnow (*Pimephales promelas*). Ecotoxicology and Environmental Safety. 140, 65-75.

3.1 Introduction

The release of contaminants, both intentional and unintentional, into the aquatic ecosystems is one of the most serious consequences of modern anthropogenic activities. Contaminants such as

metals represent significant risks due to their persistence and lack of degradation in the environment (Meyer et al. 2005, Weir et al. 2016). Our current understanding of the toxicological implications of metal exposure in fish is mainly based on studies conducted with single metal(s), even though in the natural environment organisms inhabiting metal-contaminated natural environments are almost always exposed to metals in mixtures (Borgmann et al. 2008, Vijver et al. 2011). Field based investigations have suggested that exposures to metal mixtures can lead to a myriad of adverse physiological consequences in fish including a decrease in survival and reproductive capacity (Pyle et al. 2005, Weber et al. 2008). Metals in mixtures may interact with each other because of their common pathways of uptake and metabolism, ultimately causing less than additive or more than additive toxicity in exposed organisms (Daka and Hawkins 2006).

Due to similar natural and anthropogenic release processes, cadmium (Cd) and zinc (Zn) are known to exist together at elevated concentrations in contaminated natural waters (Thornton 1992, Knapen et al. 2004, ATSDR 2008). Waterborne Cd is known to compete with calcium (Ca) for uptake in the gill and cause toxicity in fish essentially by disrupting Ca homeostasis (hypocalcemia) during acute exposure (Niyogi and Wood 2004). It has also been found to induce a wide range of adverse pathophysiological and toxicological effects during chronic exposure, including decreased growth and survival (Hansen et al. 2002, Mebane et al. 2008), elevated Cd accumulation in vital organs (McGeer et al. 2000), and disruption of ion (Na^+ and Ca^{2+}) homeostasis (McGeer et al. 2000, Reynders et al. 2006) and immune capacity (Zelikoff et al. 1995), oxidative stress (Cao et al. 2010), tissue and skeletal damage (Wangsongsak et al. 2007, Benaduce et al. 2008), and endocrine disruption (Foran et al. 2002, Lizardo-Daudt and Kennedy 2008). Moreover, a few previous studies have reported decreased spawning frequency and fecundity in fish during chronic exposure to waterborne Cd, indicating its reproductive toxicity (Beniot et al.

1976, Sellin and Kolok 2006, Wang et al. 2014). The precise mechanisms by which Cd causes reproductive effects in fish remain poorly understood, nonetheless it has been reported to reduce vitellogenesis and steroid levels in females, and impair gametogenesis in both males and females (Brown et al. 1994, Das 1998, Olsson et al. 1995, Foran et al. 2002, Tilton et al. 2003). Chronic exposure to waterborne Cd has also been reported to decrease mRNA expression of estrogen receptors and vitellogenin in the liver and gonadotropin-releasing hormone in the brain of fish (Vetillard and Bailhache 2005, Driessnack et al. 2016).

On the other hand, Zn, unlike Cd, is an essential metal as it is a necessary component in >300 proteins which play important physiological roles in growth, vision, immune response and reproduction (Hogstrand 2012). However, it can also cause toxicity in fish when they are exposed to elevated levels of Zn. Similar to Cd, Zn is also a Ca antagonist, and during acute waterborne exposure, it causes toxicity by inhibiting branchial Ca^{2+} uptake and thereby inducing hypocalcemia (Hogstrand et al. 1995, 1996). In contrast, the chronic toxicity of Zn is not very well characterized, although chronic exposure to Zn has been found to increase tissue-specific Zn accumulation (Bradley and Sprague 1985, Hogstrand and Haux 1996), and reduce survival (Bodar et al. 2005) and growth (Mohanty et al. 2009). The reproductive effects of chronic Zn exposure has been studied sporadically, and it has been found that exposure to Zn at environmentally relevant exposure levels can decrease egg production as well as egg viability in fish (Brungs 1969, Benoit and Holcombe 1978). However, very little is known about the potential mechanisms of reproductive toxicity of Zn. It has been speculated that Zn may cause reproductive impairment by affecting vitellogenin metabolism due to the inhibition of Ca^{2+} uptake in oocytes, and/or by altering steroid hormone biosynthesis or function resulting in gonad pathology (Hogstrand 2012).

To date, the interactive effects of metals in mixture on the toxicity in fish have been studied predominantly by using short-term or acute exposures with mortality as the primary endpoint (Mebane et al. 2012, Balistrieri and Mebane 2014, Clemow and Wilkie 2015, Niyogi et al. 2015), and chronic impacts of metal mixtures have received very little attention. To this end, it is to be noted here that reproductive output (fecundity) is often considered to be the most sensitive endpoint (more sensitive than early life stage effects) in fish during chronic exposure to contaminants (Suter et al. 1987). In a recent study, we have demonstrated that chronic exposure to waterborne Cu and Cd in binary mixture can elicit greater than additive effects on reproductive output (egg production) in FHM suggesting that reproductive performance can be a useful parameter for assessing the chronic toxicity of metal mixtures (Driessnack et al. 2016). Our study also indicated that the interactive effect of Cu and Cd on reproduction was possibly mediated by the disruption of estrogenic functions and energy regulation in fish (Driessnack et al. 2016). Recent evidence suggests that Zn can ameliorate Cd accumulation in reproductive tissues (e.g., ovary), and Cd-induced oxidative stress and disruption of estrogenic functions in fish (Banni et al. 2011, Chouchene et al. 2011, 2016). This raises the possibility that the interactions of Cd and Zn during chronic exposure may elicit antagonistic (less than additive) toxicity on fish reproduction.

The current study was designed to evaluate the effects of chronic exposure to waterborne Cd and Zn, both singly and in binary mixture, on reproductive performance in fish, and also to gain insights into the potential mechanisms underlying their interactive effects. We used a 21-day FHM reproductive bioassay, where fish were exposed to ~10% of 96 hr LC_{50} of each metal *via* water, individually or in combination, in addition to the control (no metals added). The specific objectives of this study were: (i) to characterize the interactive effects of waterborne Cd and Zn on the reproductive performance of fish, (ii) to examine whether the interactions of Cd and Zn influence

the accumulation of each metal in target tissues (gill, liver, and gonad), and (iii) to evaluate how Cd and/or Zn exposure influence the hepatic mRNA expression of genes that have important reproductive and metal-detoxification functions (estrogen receptors ((ER-α and ER-β), vitellogenin (Vtg), and metallothionein (MT)) and circulating estradiol level in female fish. We hypothesized that the interactions of waterborne Cd and Zn would elicit less than additive effect on the reproductive output of FHM.

3.2. Materials and methods

3.2.1. Experimental design and setup

The FHM reproductive bioassay was conducted at the Aquatic Toxicology Research Facility (ATRF) of the University of Saskatchewan. The experimental designs and fish care procedures were approved by the University of Saskatchewan Animal Care Committee and met Canadian Council of Animal Care protocols. Adult FHMs (6-9 months old) were selected at random from an in-house culture, maintained at the ATRF. Prior to the selection of fish used in the reproductive bioassay, they were reared in 400-L flow-through tanks supplied with dechlorinated municipal Saskatoon water (Ca^{2+} 44, Mg^{2+} 18, Na^{+} 26, K^{+} 3, Cl^{-1} 11, SO_4^{-2} 50, hardness 155, alkalinity 110 (both as $CaCO_3$), dissolved organic carbon (DOC) 2.5 (all in mg/L), pH 7.9). The methodology used to analyze the water quality parameters are described elsewhere (Driessnack et al. 2016).

Exposure to metals was based on a full-factorial two-way study design and consisted of the following 4 treatments: (i) control (dechlorinated Saskatoon municipal water; no added Cd or Zn), (ii) waterborne Cd only (6µg/L, added as $CdCl_2$ (Sigma-Aldrich, MO, USA)), (iii) waterborne Zn only (200 µg/L, added as $ZnCl_2$ (Sigma-Aldrich, MO, USA)), and (iv) binary mixture of

waterborne Cd and Zn (6 and 200 µg/L, respectively). The concentrations of Cd and Zn mentioned here represent nominal concentrations and equate to ~10% of 96 hr LC_{50} for Cd and Zn, respectively, for FHM under ambient water chemistry (unpublished data). The measured values for Cd and Zn exposure concentrations are reported in the results section (see Table 3.1).

The FHM partial lifecycle reproductive assay was performed following the methodology described in our previous studies (Ouellet et al. 2013, Driessnack et al. 2016), modified from the original assay described by Ankley et al. (2001). To perform this assay, an enclosed proportional diluter system was set up using 25 individual 9-L glass aquaria (six replicates for each metal(s) treatment, and seven replicates for control). Each aquarium contained one PVC breeding tile and one FHM breeding trio (1 male: 2 female). Treatment water dilutions were prepared using a proportional dual head metering pump (Q2V, Fluid Metering Inc., NY, USA) drawing at a fixed rate from a control water head tank and the corresponding metal treatment head tank. Water from each tank was mixed within a pressurized manifold system (JCM Specialties, SK, Canada), which delivered the appropriate treatment water to the corresponding aquaria. The metering pumps were set to draw at a rate of 3 turnovers per day for each aquarium. All of the aquaria were maintained at 25 ± 2°C using a temperature-controlled water bath, and the light cycle was set to 16 hr:8 hr (light: dark).

3.2.1.1 Pre-exposure period

A 21-day pre-exposure phase was carried out to ensure that each FHM trio (n = 48 trios) selected for the exposure phase demonstrated an established baseline level of reproductive output prior to being exposed to metal(s). Criteria employed to determine the stability of FHM trio basal reproductive output was: 100% survival of adult FHMs, egg fertilization > 80%, successful

spawning events every 2-3 days, and trios producing approximately 10-20 eggs/female/day (OECD 2003, Ouellet et al. 2013, Driessnack et al. 2016). Prior to the placement in pre-exposure aquaria, each fish was evaluated for fork length, weight, and secondary sex scoring. All FHM trios used in the pre-exposure and exposure phases of the experiment were fed a commercially available frozen bloodworm diet (Sally's Bloodworms^{TM}, San Francisco Bay Brand Inc., Newark, CA, USA), twice daily at approximately 10-12% of combined body weight (~1g/trio). Approximately 10-15 minutes following each feeding episode, all aquaria were cleaned with a fine mesh net to remove the uneaten food and feces. Additionally, walls of the aquaria, tubing, air stone, outflow standpipe and spawning tiles were cleaned every other day for the entire duration of the pre-exposure and exposure phases with treatment specific cleaning tools.

Assessment of daily egg production was performed by the removal and visual examination of spawning tiles for deposition of eggs. Deposited broods that consisted of 10 or more water hardened eggs were counted as successful spawning events. Successful broods were collected by carefully removing the eggs from the spawning tile, and were then transferred to a clean petri dish at which time they were photographed. Brood photographs were used to determine the number of eggs, egg size (as the diameter (mm)) using Image J (imagej.nih.gov) and fertilization success. After broods had been photographed, they were transferred to aerated individual incubation cups containing control water. Eggs were maintained in the incubation cups to determine hatching success, time to hatch, 5-day post hatch larval survival and deformity prevalence. These procedures for the collection, assessment, and maintenance of broods were performed in the same manner throughout pre-exposure and exposure phases of the experiment.

Of the 48 trios used in the pre-exposure phase, 26 trios met the pre-established baseline reproduction criteria. From those 26 trios, 25 trios with the greatest mean egg production were

selected for use in the exposure phase. A one-way analysis of variance (ANOVA) was performed on the 25 trios chosen to verify that there were no statistical differences in mean eggs, eggs/female/day (e/f/d), fertilization success, and a Chi-Square test was used to compare the number of spawning events. No statistically significant differences were noted for mean eggs, e/f/d, fertilization success, and number of spawning events among the selected FHM trios ($p \leq 0.05$).

3.2.1.2. Exposure phase

Immediately following the completion of the pre-exposure phase, 25 FHM trios were selected randomly and placed into the appropriate exposure aquaria, and maintained for 21-days. As described in the pre-exposure phase, spawning tiles were evaluated daily for deposition of eggs. Mean eggs per FHM trio in each treatment were estimated by dividing the total eggs produced over 21-days by the number of FHM trios used. For each brood, ten eggs were randomly selected for the evaluation of egg size using Image Pro Plus 6.1 (Media Cybernetics Inc., MD, USA). Collected eggs were also evaluated for fertilization success, hatching success, time to hatch, and 5-day larval survival and deformities. Eggs and larvae were maintained separately in control water (no metals added). Water samples (2-mL) were collected daily from an aquarium selected randomly from each treatment for the determination of total metal levels. Upon completion of the 21-day exposure phase, fish were anesthetized with AquaCalm (Syndel, BC, Canada), and assessed for fork length (mm), total body weight (g), and secondary sex characteristics (nuptial tubercles, body banding, and fin dot). Blood samples were collected from females by caudal severance for the estimation of circulating estradiol level. For the measurement of tissue-specific metal accumulation, gills, liver, ovary, and carcass were dissected out from a female in each trio, rinsed for 20-s in deionized water, blotted dry, and placed in pre-weighed polythene tubes. The

tubes were re-weighed to determine the tissue weight to the nearest 0.01 mg. A portion of the ovary was collected and preserved separately for the analysis of ovarian histopathology. Additionally, the liver from the second female in all trios was collected and immediately placed in a pre-weighed vial and preserved with RNAlater (Life Technologies, ON, Canada) for the gene expression analysis.

3.2.2. Experimental analysis

During both pre-exposure and exposure phases, one aquaria per treatment was selected at random for the measurement of conductivity, dissolved oxygen, and ammonia, using methods described previously in Driessnack et al. (2016). Water samples collected for metal analysis were first passed through a 0.45 µm syringe filter, acidified (0.2% HNO_3, trace metal grade, VWR, ON, Canada) and then stored at 4 °C until analyzed. Fish tissue samples collected for measuring metal accumulation were digested in 5 volumes of 1N HNO_3 at 60°C for 48h, and then stored at 4 °C until analyzed. Water and digested tissues samples were analyzed for Cd and Zn concentrations using graphite furnace atomic absorption spectroscopy (AAnalyst 800, Perkin-Elmer, CT, USA), with a practical detection limit of 0.2 µg/L for both metals. The quality control and assurance of metal analysis were maintained using appropriate method blanks and certified standards for Cd and Zn (Fisher Scientific, ON, Canada). In addition, the efficiency of Cd and Zn analysis in fish tissue samples was evaluated by analyzing a certified reference material (DOLT-3; National Research Council, Canada). The recovery of Cd and Zn in the reference material was 96% and 93%, respectively.

Fathead minnow blood samples were kept on ice immediately after collection, and then centrifuged at 2,000 g for 3 minutes to isolate plasma. The plasma estradiol quantification was

done using a commercial ELISA kit (Cayman Chemical, MI, USA), following the instructions provided by the supplier. Histopathological analysis was performed on ovaries that were fixed in 4% paraformaldehyde (PFA) for 5 hrs and then stored in 70% ethanol until processing. An automated tissue processor (MUP1, Modular Vacuum Processor) was used to process the ovarian tissues. Briefly, tissues were dehydrated through a series of graded ethanols (70-100%) and perfused with paraffin wax, as recommended in established protocols (US EPA, 2006; OECD, 2009). Immediately following processing, tissues were manually fixed in paraffin. The paraffin blocks were then sectioned into 5-7 μm thick sections using a rotary microtome (HM330; Heidelberg, Germany). Tissue sections were fixed on pre-cleaned suprafrost slides (VWR, ON, Canada), and dried on a slide warmer. Tissues were then de-paraffinized, rehydrated and stained using Meyer's hematoxylin and eosin, following recommended protocols (US EPA 2006, OECD 2009). Once slides were stained and coverslips affixed, they were examined using a Zeiss Axioplan Fluorescence Microscope and photographed using an AxioCamICc1 (Colour, 1.4 MP) digital camera. Ovarian sections (n = 15-20 female FHMs) were photographed in 2-3 non-overlapping areas for the identification and estimation of different follicle stages. The distribution of FHM oocytes is random within the ovary. Therefore each section is considered representative of the whole ovary (Wolf et al. 2004). For each ovarian section, an average 50 follicles were counted and determined to be either: previtellogenic (primary oocytes; cortical alveolar), post-vitellogenic (early or late vitellogenic; mature), or atretic (underdeveloped or degenerating). Different types of follicles were estimated as a proportion of total follicles (e.g., number of previtellogenic follicles per total follicles counted), as described in previous studies (Miles-Richardson et al. 1999, Wolf et al. 2004, Leino et al. 2005). Examination of FHM ovarian sections was completed using Image J (imagej.nih.gov).

For gene expression analysis, first the extraction of RNA was conducted from the liver samples preserved in RNALater. For this, samples were homogenized using a handheld tissue homogenizer (VWR International, ON, Canada) and RNA was extracted using the illustra RNAspin Mini Kit (GE Healthcare). Once purified RNA was obtained, nucleic acid content and quality was quantified using a NanoDrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA); samples were then stored at -80°C until further processing. cDNA was synthesized from the RNA samples using a qScript cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, MD, USA) and manufacturer's instructions. It consisted of combing 4µl of qScript Reaction Mix (5X), 1 µl of qScript RT, and calculated amounts of RNA (values obtained from NanoDrop) and nuclease-free water to obtain a sample concentration of 1µg/µl cDNA in a final reaction volume of 20µl. The cDNA synthesis was carried out in a thermal cycler (MyCycler, Bio-Rad Laboratories Inc.) for 1 cycle at 22 °C for 5 min, 1 cycle at 42 °C for 30 min, 1 cycle at 85 °C for 5 min then held at 4 °C until stored at -20 °C. The primers of genes analyzed in the present study (ER-α, ER-β, Vtg, MT and β-actin (used as a housekeeping gene)) were synthesized by Integrated DNA Technologies (IDT, IA, USA). All gene primer sequences have been used in our previous studies (Driessnack et al. 2016). After the reaction conditions for each gene were optimized, standard curves were run in duplicate for individual gene, to determine linearity, detection range and efficiency. Standard curves were also run using pooled control female FHM liver cDNA samples, over a 10-fold serial dilution series in triplicate.

RT-PCR was initiated upon successful completion of the optimization and generation of standard curves for each gene. RT-PCR analysis was completed using an ABI 7300 Real-Time PCR System (Applied Biosystems, Life Technologies, ON, Canada) and a SYBR® Green PCR Master Mix (Applied Biosystems) with a ROX dye. All female liver cDNA samples were run in

triplicate, in addition to a no template negative control, with a final reaction volume of 25 µl PCR reaction volume consisting of 12.5 µl of 2X SYBR® Green Master Mix, 2.0 µl of cDNA, and optimization derived volumes of $MgCl_2$, reverse and forward primers for each gene. All samples were run using 96-well plates and proceeded through PCR cycling conditions set by manufacturer's protocol. The conditions recommended by the manufacturer were comprised of an initial denaturation step of 10 min at 95 °C to activate AmpliTaq Gold®, followed by 40 cycles of denaturation at 95 °C for 15 seconds and annealing at 60 °C for 60 seconds. Target gene expression values were normalized to values obtained for β-Actin expression and evaluated using a 2-ΔΔCT approach (Schmittgen and Livak 2008). The changes in the gene expression levels are reported as the average change in transcript abundance per treatment.

3.2.3. Statistical analyses

All statistical tests were completed using the statistical program SPSS 22.0 (SPSS, Chicago, Il, USA). Before any statistical testing, all data were evaluated for normality (Kolmogorov-Smirnov (K-S) test) and homogeneity of variance (Levene's test) to determine if parametric or non-parametric tests should be employed. Data that failed to meet the assumptions of normality or equal variance were first transformed (log_{10} +1 or arcsin for percent data) and re-assessed. If log transformation of the data still did not meet the assumptions for parametric testing, a non-parametric extension of the 2-way ANOVA, the Scheirer-Ray-Hare test was performed. A 2-way ANOVA or the Scheirer-Ray-Hare test (when necessary) was carried out to evaluate significant differences in hatching success, fertilization success, larval deformities, larval survival, gene transcript abundance (ER-α, ER-β, Vtg, and MT), plasma estradiol concentrations, Cd and Zn levels in fish tissues (gill, liver, ovary, and carcass) and exposure water. Cumulative endpoints (spawning events and egg production over 21 days) were assessed using the K-S test to compare

the Cd-only, Zn-only and Cd-Zn mixture treatments with the control treatment. The other endpoints such as the number of spawning events per treatment, female ovipositor score and maleness index for male secondary sex characteristics and number of nuptial tubercles are all ordinal data, and were assessed using the Chi-Square test.

3.3. Results

3.3.1. Exposure concentrations of metals and water quality

The measured concentrations of Cd and Zn in different treatments have been provided in Table 3.1. The concentrations of Cd and Zn in the exposure water were significantly elevated in respective single metal treatments relative to that in the control. In the Cd and Zn mixture treatment, the exposure concentration of each metal was similar to that in their respective single metal treatments. No significant difference in water quality (conductivity, dissolved oxygen, and ammonia) was recorded among any treatments during the entire exposure duration. General water quality parameters are presented in Table A1 of the Appendix.

3.3.2. Fish morphometrics and reproductive performance

No fish mortality was recorded in any treatments during the exposure phase. There was no difference in fish weight (male and female), length, condition factor, and secondary sexual characteristics among different experimental treatments (data not shown). Similarly, there were no differences in female hepatosomatic index (HSI) or gonadosomatic index (GSI) among any treatments (Table 3.2).

Cumulative egg production over 21 days was significantly decreased (a 46% reduction) by co-exposure to waterborne Cd and Zn relative to the control, while egg production was significantly affected by exposure to Zn alone, but not by exposure to Cd alone (Table 3.2).

Table 3.1: Measured dissolved metal concentrations of Cd and Zn in different treatment waters. Values are presented as mean ± SEM (n = 21). The asterisk (*) represents a significant difference in Cd concentration relative to control. Significant differences in Zn among the four treatments are indicated by different letters.

Treatment	Cd (µg/L)	Zn (µg/L)
Control	< d.l	5.12 ± 0.36 [a]
Cd-Only	7.67 ± 0.77 *	8.05 ± 0.26 [a]
Zn-Only	< d.l	173.50 ± 12.54[b]
Cd-Zn	7.22 ± 0.37 *	170.90 ± 13.62[b]

Table 3.2: Morphometrics and reproductive performance in adult fathead minnow (FHM) following 21-day exposure in different experimental treatments. Data are presented as mean ± SEM (n = 6-7 trios). Different letters denote statistically significant differences among the four treatments ($p \leq 0.05$). All endpoints were assessed using a 2-Way ANOVA unless denoted with a "-*," indicating statistical analysis used was the Kolmogorov-Smirnov Test.

Endpoint	Control	Cd-only	Zn-only	Cd-Zn
Male GSI	1.10 ± 0.24[a]	0.76 ± 0.17[b]	1.19 ± 0.20[a]	1.53 ± 0.18[a]
Female HSI	1.98 ± 0.50[a]	2.52 ± 0.27[a]	1.95 ± 0.45[a]	1.82 ± 0.44[a]
Female GSI	9.71 ± 2.54[a]	4.40 ± 0.90[a]	10.46 ± 2.00[a]	10.46 ± 1.71[a]
Cumulative Total Eggs[*]	947[a]	1467[ab]	730[b]	512[c]
Mean Eggs/trio	50.05 ± 17.4[a]	86.29 ± 66.4[a]	34.33 ± 16.5[a]	33.47 ± 17.1[a]
Spawning events/trio	3.2 ± 0.5[a]	2.7 ± 0.6[a]	3.7 ± 0.9[a]	2.7 ± 0.6[a]
Fertilization Success (%)	99.73 ± 0.18[a]	99.67 ± 0.23[a]	41.37 ± 18.86[b]	43.67 ± 20.26[b]
Time to hatch (days)	6.0 ± 0.4[a]	6.3 ± 0.7[a]	6.2 ± 0.1[a]	6.2 ± 0.2[a]
Egg Size (mm)	1.42 ± 0.01[a]	1.41 ± 0.01[a]	1.40 ± 0.01[a]	1.42 ± 0.01[a]

Nonetheless, the egg production in the Zn alone treatment was significantly lower when compared to that in the Cd alone treatment. No significant differences were recorded for mean eggs and spawning events per FHM trio among the experimental treatments (Table 3.2). Exposure to waterborne Zn, alone and in mixture with Cd, decreased fertilization success (%) by the same magnitude (more than 2-fold) relative to the control. However treatment with waterborne Cd alone did not affect, fertilization success (Table 3.2). Time to hatch, size of the eggs (Table 3.2), hatching success, and incidences of deformities (Table A2 of appendix) were not affected by exposure to waterborne Cd and/or Zn.

3.3.3. Hepatic gene expression

Female FHMs were analyzed for hepatic expression of reproductively important genes (ER-α, ER-β, and Vtg) as well as the metallothionein (MT) gene. The transcript abundance of both estrogen receptors (ER-α and ER-β) was significantly elevated, compared to the control, following exposure to waterborne Zn, but not to waterborne Cd (Fig. 3.1A and B). In contrast, exposure to the mixture of waterborne Cd and Zn resulted in a significant reduction of ER-α and no change in ER-β transcript abundance relative to that in the control (Fig. 3.1A and B). The hepatic mRNA expression of Vtg decreased significantly following treatments with Cd or Zn, alone and in mixture, relative to the control, and no difference was recorded among the three experimental treatments with metal(s) (Fig. 3.1C). The transcript abundance of hepatic MT was not affected by exposure to waterborne Cd alone relative to the control. However exposure to Zn, alone and in combination with Cd, resulted in a significant increase in hepatic mRNA expression of MT (Fig. 3.1D). The increase in MT gene expression in the latter two treatments was modest (2-4 fold) and did not differ significantly from each other. No significant interaction of Cd and Zn was noted for the expression of MT gene as well.

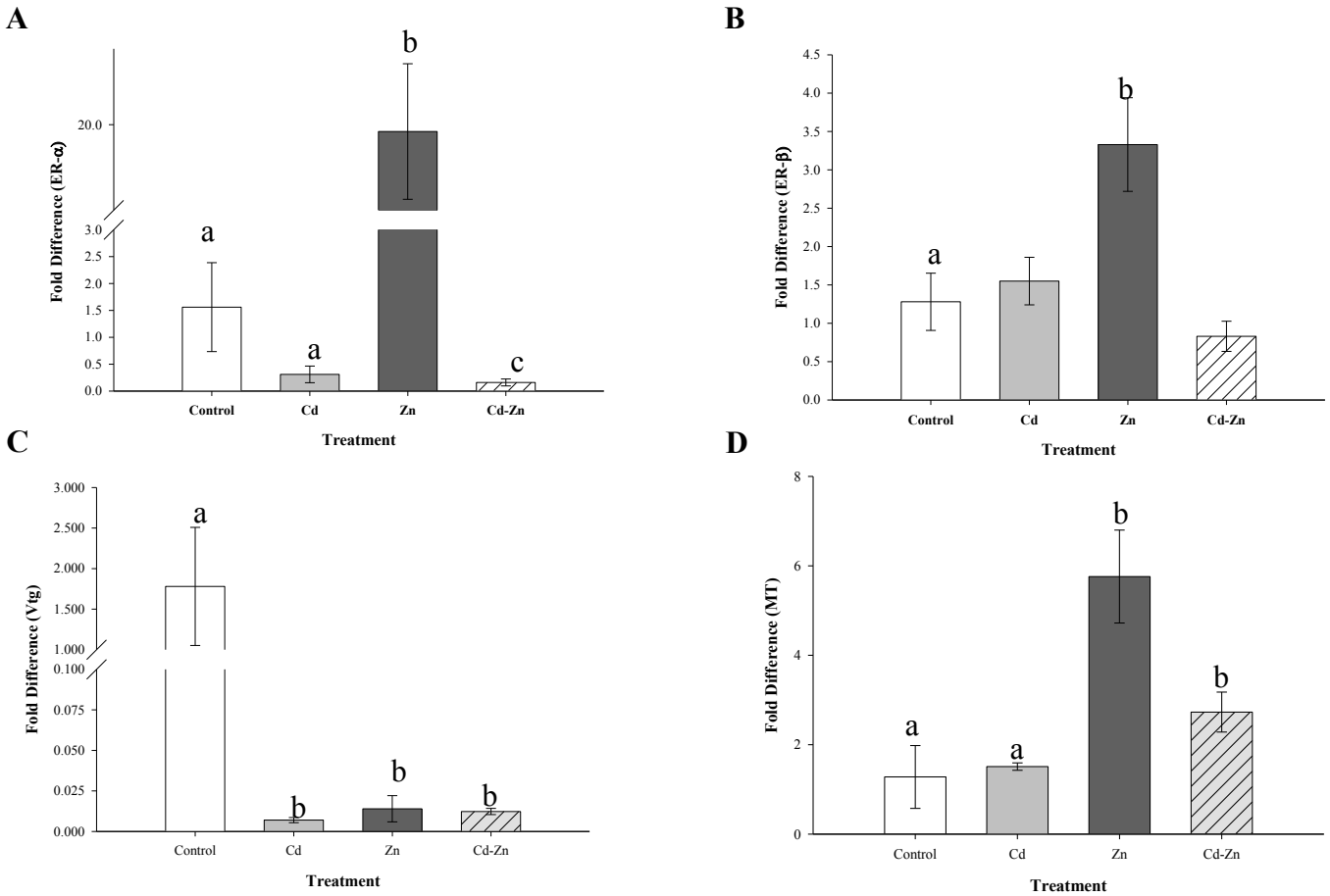

Figure 3.1 Female fathead minnow hepatic mRNA expression for estrogen receptor (ER-α (A) and ER-β (B)), vitellogenin (Vtg) (C), and metallothionein (MT) (D) following 21-days of waterborne exposure to Cd and Zn, individually and in binary mixture. Data are shown as mean transcript abundance ± SEM (n = 4-5). Transcript abundance is expressed relative to the reference gene, β-actin. Significant differences ($p \leq 0.05$) among treatments are denoted by different letters following analysis by 2-way ANOVA or Scheirer-Ray-Hare (Vtg and MT).

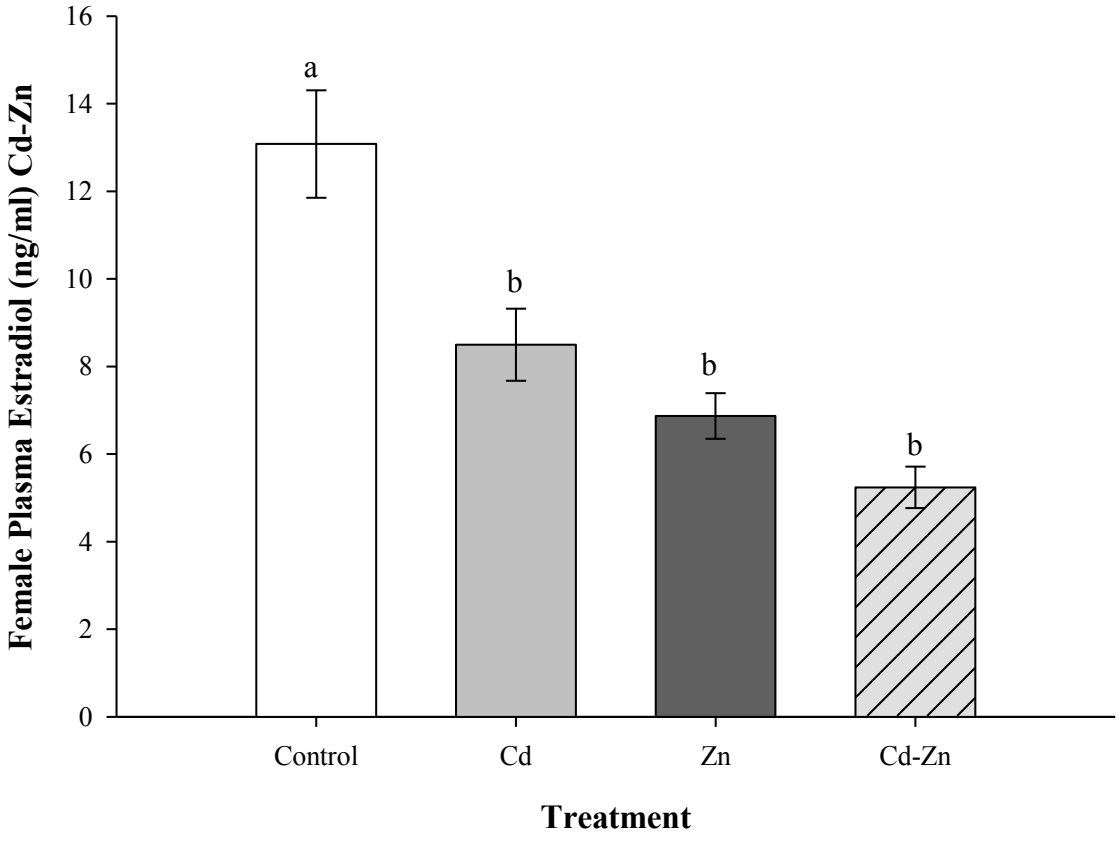

Figure 3.2: Female fathead minnow plasma estradiol concentrations following 21-day waterborne exposure to Cd and Zn, alone and in combination. Data are depicted as mean ± SEM (n = 4-5). Data was analyzed using a 2-Way ANOVA with significant differences ($p \leq 0.05$) among treatments being identified with different letters.

3.3.4. Plasma estradiol levels and ovarian histology

The circulating estradiol level was significantly reduced in female FHM following exposure to waterborne Cd and Zn, both alone and in mixture relative to that in the control fish (Fig. 3.2). The magnitude of reduction was significantly higher (62% decrease compared to the control) in fish co-exposed to waterborne Cd and Zn relative to that in fish exposed to waterborne Cd or Zn alone (30-42% decrease compared to the control).

The standardized histopathological assessment of ovary indicated no significant change in the proportion of pre-vitellogenic or post-vitellogenic follicles among any of the experimental treatments. However, a significant increase in follicular atresia (underdeveloped or degenerating follicles) was recorded in the ovary of fish exposed to waterborne Cd and Zn mixture, but not to Cd or Zn alone (Fig. 3.3).

3.3.5. Tissue-specific metal accumulation

The accumulation of Cd and Zn was analyzed in the gill, liver, ovary, and carcass of female fish. For both Cd and Zn, tissue concentrations were highest in the liver followed by gill, carcass, and ovary. Exposure to waterborne Cd, alone and in mixture with Zn, resulted in a significant increase in Cd accumulation in all four tissues relative to the control (Fig. 3.4A - D). However, Cd burden in the gill and liver of fish co-exposed to waterborne Cd and Zn was significantly lower (2-3 fold) than that in fish exposed to waterborne Cd alone (Fig. 3.4A and B). This decrease in Cd burden following 21days of exposure to the mixture of waterborne Cd and Zn was not observed though in the ovary or carcass (Fig. 3.4C and D). The tissue-specific Cd accumulation in fish exposed to waterborne Zn alone did not differ from that in the control fish.

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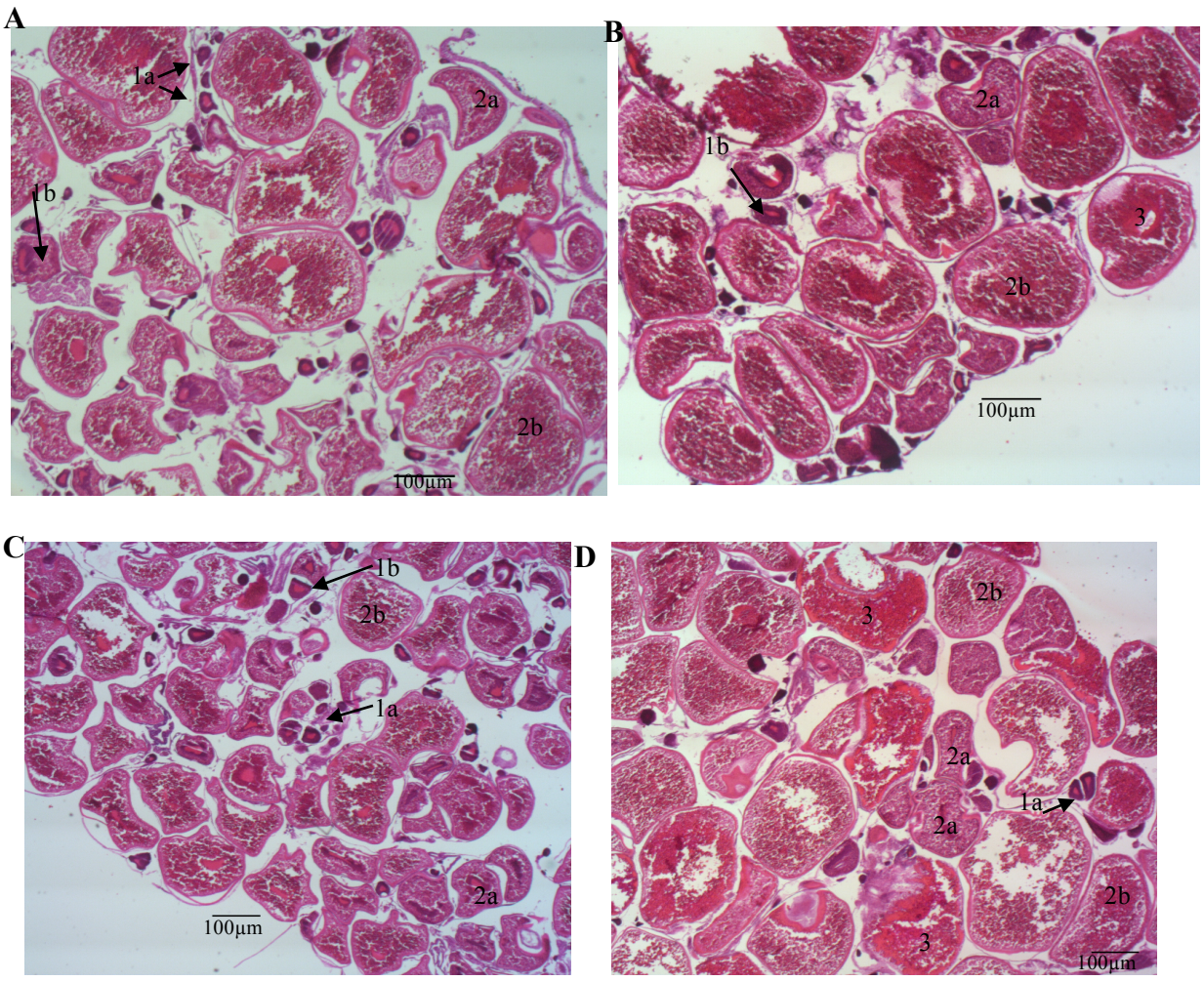

Figure 3.3: Part A: Fathead minnow ovarian histological sections following 21-day waterborne exposure to either Cd and Zn, singularly and in binary combinations. Ovarian tissues processed with paraformaldehyde/paraffin method, sectioned at 5-7 µm, and H&E staining. (A) Control ovarian section. Shown are 1a (perincucleolar oocyte) and 1b (cortical alveolar) representing follicles in the pre-vitellogenic stage, 2a (early vitellogenic) and 2b (late vitellogenic) indicating follicles in the post-vitellogenic stage. (B) Cd-only exposed ovarian sections. Shown is a follicle indicating the atresia stage (3). (C) Zn-only exposed ovarian sections with different follicular stages (1 & 2) indicated. (D) Cd-Zn exposed ovarian section

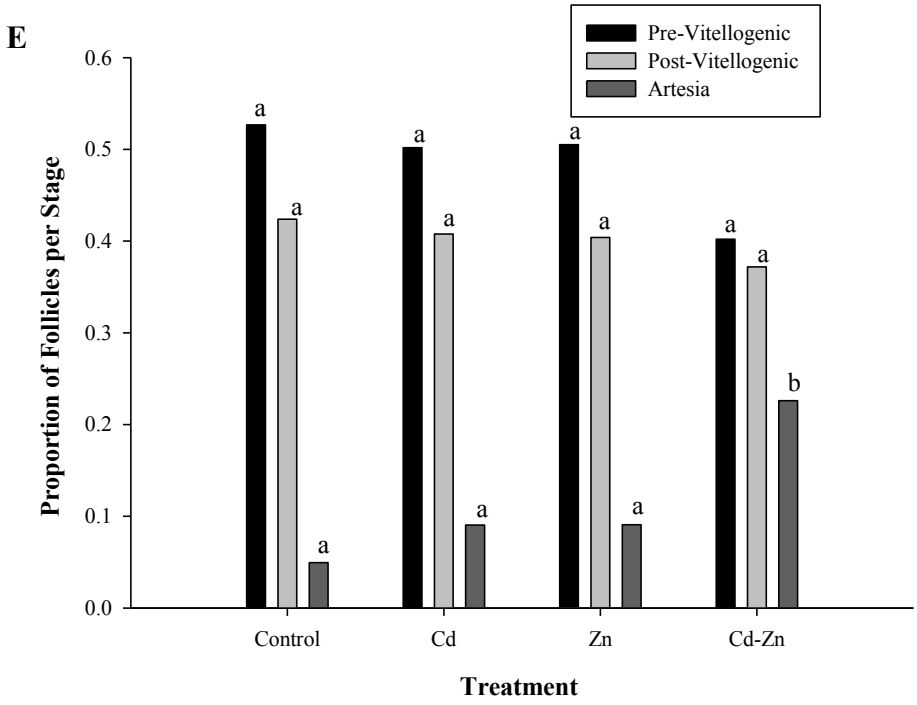

Figure 3.3 Part B: (E) Relative proportion of cells per follicular stage in the ovaries of female fathead minnows following 21-day waterborne exposure to Cd and Zn, alone and in combination. Analysis was completed using 2-Way ANOVA, with *-denoting significant differences between treatment stages.

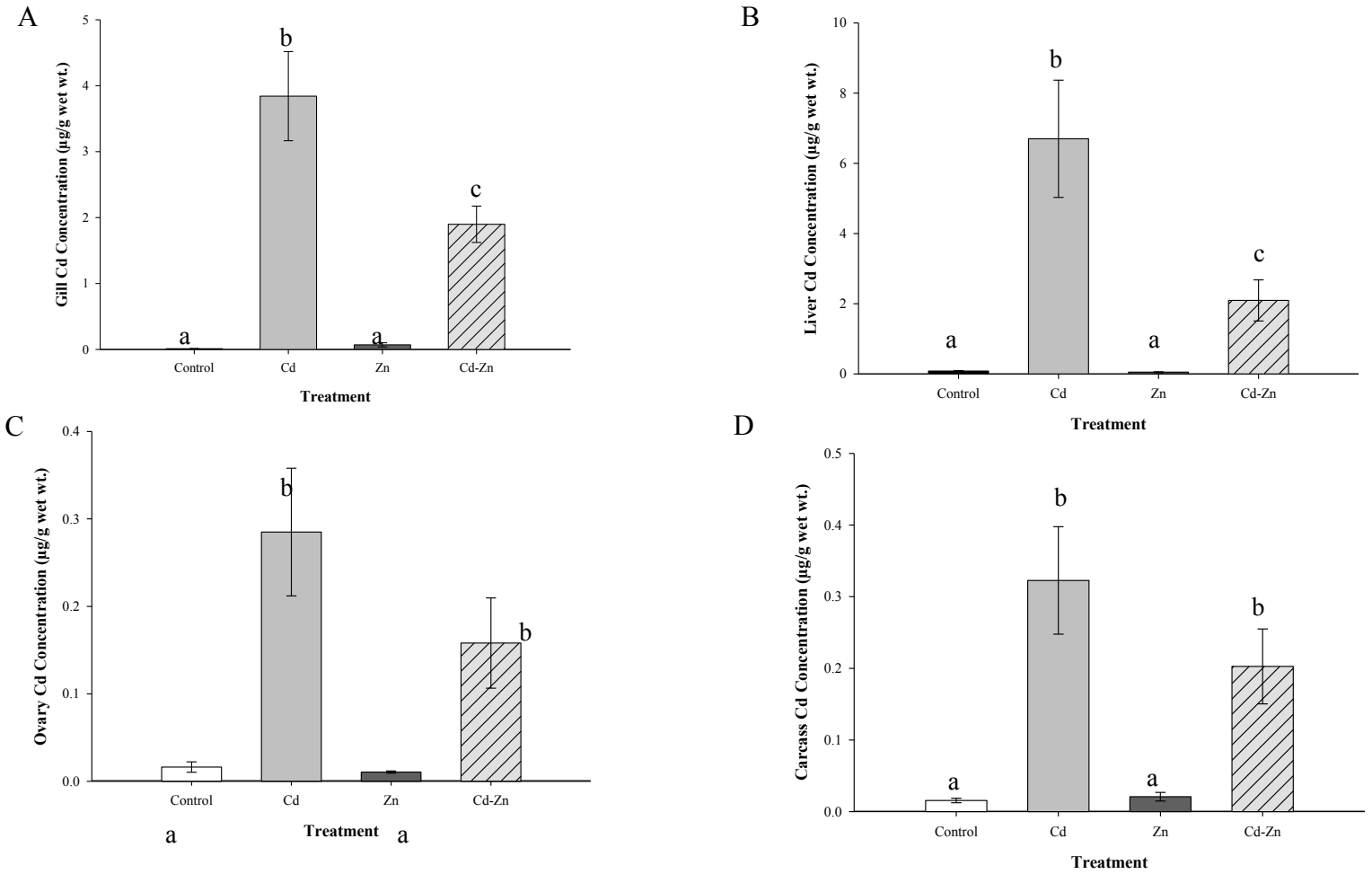

Figure 3.4: Tissue-specific Cd concentrations in fathead minnows following 21-days of waterborne exposure to Cd and Zn, singularly and in binary combination. Data are shown as mean ± SEM, gill (A) and carcass (D) are for males and female (n = 18-20) and ovary (C) and liver (B) for females only (n = 6-7). Significant differences ($p \leq 0.05$) among treatments are indicated by different letters.

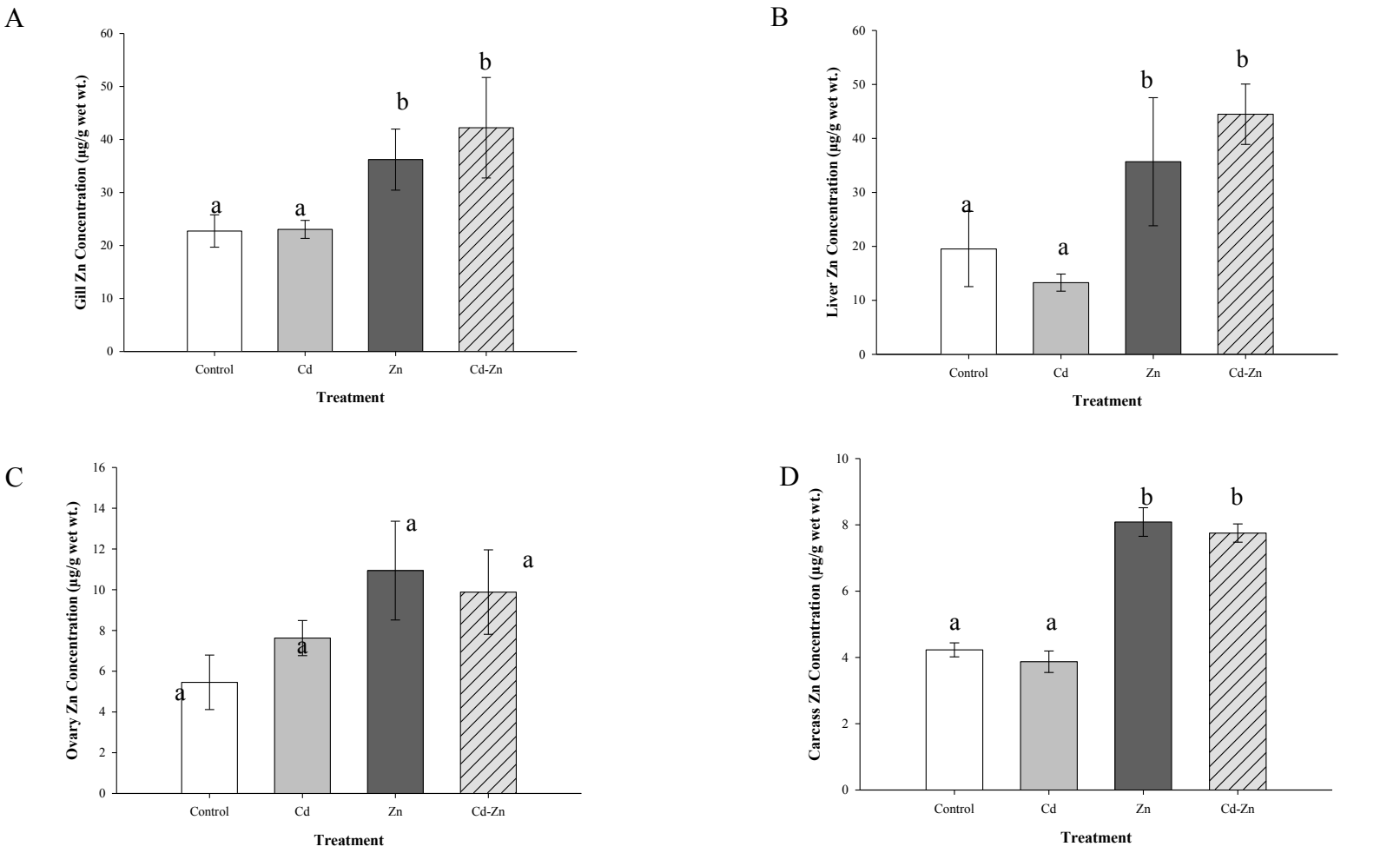

Figure 3.5: Tissue-specific Zn concentrations in fathead minnows following 21-days of waterborne exposure to Cd and Zn, singularly and in binary combination. Data are shown as mean ± SEM, gill (A) and carcass (D) are for males and female (n = 18-20) and ovary (C) and liver (B) for females only (n = 6-7). Significant differences ($p \leq 0.05$) among treatments are indicated by different letters.

As observed with tissue-specific Cd accumulation, exposure to waterborne Zn, alone and in combination with waterborne Cd, led to a significant increase in Zn accumulation in the gill, liver, and carcass (Figure 3.5A - C). However, no significant change in the ovarian Zn concentration was recorded among any of the experimental treatments (Fig. 3.5D). In addition, co-exposure to Cd and Zn did not alter the Zn levels in any tissues relative to the Zn only exposure, and no significant interaction between Cd and Zn was noted. Zinc levels in all four tissues in fish exposed to waterborne Cd alone remained similar to that in the control fish.

3.4. Discussion

The concentrations of Cd and Zn used in the waterborne exposures of the present study have often been reported in contaminated aquatic ecosystems (ATSDR 2008, Luoma and Rainbow 2008). Thus, contrary to our original assumption, the present study demonstrated that the interactions of Cd and Zn during environmentally relevant waterborne chronic exposure could elicit greater than additive effects on fish reproductive output.

In our study, cumulative egg production in FHM was the most sensitive reproductive endpoint, which was significantly affected by co-exposure to waterborne Cd and Zn relative to the control, but not by exposure to either Cd or Zn alone (Table 3.2). Consistent with the present study, we have previously reported that waterborne Cd did not affect the reproductive performance in FHM, when they were exposed to 5 μg/L Cd under identical water chemistry conditions and experimental set-up. Moreover, Sellin and Kolok (2006) evaluated the concentration dependent reproductive effects of Cd in moderately hard water using the same FHM reproductive bioassay used in the present study. They reported a significant reduction in egg production and spawning frequency, but only at a Cd concentration of 50 μg/L, which was considerably higher than that (7

μg/L) used in the present study. Interestingly though, we recorded a significantly increased egg production in Cd only treatment relative to the Zn only treatment. In fact, it was higher than the control, albeit not statistically significant because of the high variability in egg production per female in Cd only treatment. Cadmium at low exposure levels has occasionally been found to induce a hormetic response in fish and other organisms (Weis and Weis, 1986; Zhang et al., 2009), and the same phenomenon might have occurred in the present study.

On the other hand, exposure to waterborne Zn alone reduced FHM egg production by 23% relative to the control, but it was not statistically significant. Brungs (1969) previously reported a significant decrease in egg production in FHM exposed to 180 μg/L in moderately hard water, which is comparable to the Zn exposure level (~170 μg/L) employed in our study. However, it is to be noted here that Brungs (1969) used an exposure period of 10 months (a full life-cycle bioassay), whereas we used a 21-day exposure period (a partial life cycle). Similar to our observation, Spehar (1975) also reported a decreasing trend, although not statistically significant, in reproductive performance (spawning per female, and embryo production) in flagfish (*Jordanella floridae*) exposed to ≥139 μg/L waterborne Zn over 45 days. In addition, we found that fertilization success in FHM was reduced by the same magnitude during exposure to waterborne Zn, alone and in mixture with Cd (Table 3.2), but not by waterborne Cd alone, thereby suggesting that the effect was essentially induced by Zn exposure. Benoit and Holocombe (1978) suggested that exposure to waterborne Zn (≥145 μg/L) affects adhesiveness and fragility of FHM eggs, which occurs shortly after the eggs are spawned, and this might have led to the reduction in fertilization success observed in our study.

Although exposure to waterborne Zn or Cd alone did not affect FHM egg production in the present study, co-exposure to both metals, each at a concentration comparable to the individual exposure, decreased egg production ~50% (significant, $p < 0.05$) relative to the control. This suggests that waterborne Cd and Zn in mixture may induce a greater than additive effect on fish reproduction. To the best of our knowledge, the chronic effects of waterborne Cd and Zn in binary mixture on fish reproduction has been examined only once before by Spehar et al. (1978). They observed that co-exposure of Cd (4-8 μg/L) and Zn (73-139 μg/L) generally elicited greater effects on the reproductive performance (mean spawning per female, and embryo production), than the individual exposure of each metal, in flagfish. To date, the interactions of waterborne Cd and Zn in aquatic organisms have been studied mainly under short-term (e.g., 96 h) acute exposure scenarios, which have indicated that the toxicity of Cd and Zn in mixture is influenced by their dose ratio in the mixture (Mebane et al. 2012, Meyer et al. 2015a, 2015b, Niyogi et al. 2015). Therefore, future studies should evaluate how the chronic toxicity of waterborne Cd and Zn mixture is modulated by the alteration of their relative concentrations in the exposure.

The sex steroid hormone estradiol is known to play a critical role in regulating reproductive output in fish. Vitellogenesis, which is an important process for the development and maturation of eggs in fish, is triggered by the binding of circulating estradiol to the estrogen receptors located in hepatocytes (Nelson and Habibi 2010). Estradiol first binds to the estrogen receptor ER-β, which induces vitellogenin synthesis. It also increases the expression of the other estrogen receptor, ER-α, resulting in hepatocytes being sensitized further to stimulation by estradiol, thereby producing more vitellogenin (Nelson and Habibi 2010). In the present study, the circulating estradiol level in female FHM was significantly reduced following exposure to both waterborne Cd and Zn individually (Fig 3.2). In previous studies, we did not record any significant change in the plasma

estradiol in FHM exposed to 1-5 μg/L Cd (Wang et al. 2014, Driessnack et al. 2016), which were slightly lower than the Cd exposure level used in the present study (7 μg/L). Nonetheless, a reduction in plasma estradiol was reported in Japanese medaka (*Oryzias latipes*) during chronic exposure to 10 μg/L of waterborne Cd (Tilton et al. 2003). Cadmium has been found to inhibit steroid synthesis and release in fish (Sangalang and O'Halloran 1972, Tilton et al. 2003), and it has been suggested that this occurs due to Cd-induced disruption of Ca signal transduction mechanisms in the pituitary and gonad, and/or inhibition of enzymes (e.g., CYP450) responsible for steroid production (Thomas 1999, Le Guével et al. 2000). There has been no previous report of reduced plasma estradiol in fish following chronic exposure to waterborne Zn. Nonetheless Zn like Cd is a Ca antagonist (Hogstrand 2012) and thus may also affect steroid synthesis by disrupting Ca-signaling pathways. Evidence of Zn-mediated disruption of Ca signaling pathways has been reported in mammalian systems (Traboulise et al. 2007, Pitt and Stewart 2015). Interestingly, however, we observed in the present study that exposure to waterborne Cd and Zn in mixture produced a greater reduction in plasma estradiol level relative to Cd or Zn only exposure (Fig. 3.2). This may have occurred because of the additive interaction of Cd and Zn on the pathways that regulate estradiol synthesis and/or release in female fish.

Cadmium is known to be a metalloestrogen as it can activate the estrogen receptors by binding to them (Martin et al. 2003). However, we did not observe any change in the hepatic mRNA expression of either ER-α or ER-β in female FHM exposed exclusively to waterborne Cd (Fig. 3.1A and B). Previous studies that examined the effects of chronic exposure to waterborne Cd (5-10 μg/L) on fish reproduction have also reported either no change in the hepatic expression of estrogen receptors or a modest upregulation of ER-β (Tilton et al. 2003, Driessnack et al. 2016). In contrast to Cd, chronic exposure to Zn alone resulted in a significant increase in the hepatic

expression of both ER-α and ER-β relative to the control (Fig. 3.1A and B). At present, it is not clear how exposure to Zn can activate the estrogen receptors, and no previous studies have examined the effects of Zn exposure on the expression of estrogen receptors in sexually mature fish. Mammalian studies suggest that although Zn can bind to the estrogen receptor, it does not lead to the activation of the receptor (Medici et al. 1989, Martin et al. 2003). Nonetheless, we observed a significant downregulation of ER-α and no change in ER- β in female FHM exposed to waterborne Cd and Zn in mixture relative to the control (Fig. 3.1A and B). Based on the individual effects of Cd and Zn on the estrogen receptor expression in the present study, it does not appear that the downregulation of ER-α in the metal mixture treatment occurred due to the binding of Cd and/or Zn to the estrogen receptors. Rather, it was probably mediated by the decrease in the circulating estradiol level (Nelson and Habibi 2010), which was lowest in the metal mixture treatment relative to the control as well as treatments with individual metals (Fig. 3.2). Overall, our findings indicate that hepatic estrogen receptors in female FHM are not particularly sensitive to Cd and/or Zn, at least not at the exposure levels used in our study.

Irrespective of the changes in the expression of estrogen receptor genes, the hepatic expression of the Vtg gene was markedly decreased in female FHM following exposure to waterborne Cd and/or Zn in the present study (Fig. 3.1C) – indicating a downregulation of vitellogenesis. This might have been caused by the negative feedback of decreased circulating estradiol level (Nelson and Habibi 2010). Interestingly though, a decrease in hepatic Vtg expression did not translate into the reduction of egg production except in fish co-exposed to waterborne Cd and Zn. In our previous study, we also found that a downregulation of hepatic expression of Vtg gene but no concomitant decrease in egg production in FHM exposed to waterborne Cd alone (Driessnack et al. 2016). Our present study also revealed an increased

incidence of follicular atresia in the ovary of FHM when they were exposed to Cd and Zn in mixture, but not individually (Fig. 3.3). It is important to note here that fish fecundity is regulated not just by the quantity but also by the quality of vitellogenin, which usually contains large amounts of Ca (Nelson and Habibi 2010). Thus, it is possible that the increase in follicular atresia and subsequent reduction in egg production in fish co-exposed to waterborne Cd and Zn might have occurred due to the disruption of Ca incorporation into the oocytes as both Cd and Zn are potent Ca antagonists (Niyogi and Wood 2004, Hogstrand 2012). Moreover, plasma estradiol has been suggested to inhibit apoptosis and atresia in fish ovaries (Janz and Van der Kraak 1997), and thus increased follicular atresia in Cd and Zn mixture treatment might also have been induced by the reduction in circulating estradiol. In general, our findings indicate that although Cd or Zn exposure alone did not cause adequate estrogenic and ovarian pathophysiological alterations to affect higher-level reproductive endpoints, their combined effects exceeded the threshold that culminates in the significant impairment of reproductive output in FHM. Contrary to our findings, co-treatment with Zn has been found to ameliorate Cd-induced disruption of estrogen signaling in larval zebrafish (*Danio rerio*) brain during short-term waterborne exposure (Chouchene et al. 2016). Similarly, Zn supplementation in the diet has been reported to partially restore histopathological changes in the ovary of zebrafish chronically exposed to waterborne Cd (400 μg/L) (Chouchene et al. 2011). Both of these studies, however, were conducted using Cd exposure levels that were not environmentally relevant (more than 50-fold higher than the present study), and thus the actual environmental significance of Cd-Zn antagonism observed in these studies is limited.

In the present study, individual exposures to waterborne Cd and Zn resulted in a significant increase in tissue-specific Cd and Zn (except in ovary) accumulation, respectively, in female FHM

(Fig. 3.4 and 3.5). Previous studies also reported a similar increases in Cd and Zn burden in target tissues of fish following chronic waterborne exposure to Cd (Driessnack et al. 2016) and Zn (Arini et al. 2015), respectively. In addition, we also observed that Cd tissue burden (gill and liver) in fish co-exposed to waterborne Cd and Zn was significantly decreased relative to fish exposed to Cd alone (Fig. 3.4A and B), although no such effect was recorded for Zn accumulation in any tissues between fish exposed to waterborne Zn alone and in combination with Cd (Fig. 3.5). The decrease in Cd accumulation in the metal mixture treatment occurred likely due to the competitive interaction of Cd and Zn at the common transport sites (e.g., epithelial Ca channels, ZIP transporters) (Hogstrand 2012, McGeer et al. 2012, Niyogi et al. 2015). On the other hand, the lack of reciprocal inhibitory effect of Cd on Zn accumulation in the metal mixture treatment could be explained based on the fact that Zn, unlike Cd, is an essential metal and thus have a much larger pool of uptake and handling sites than Cd (Niyogi et al. 2015). Arini et al. (2015) also examined the chronic interactive effects of waterborne Cd and Zn on tissue burden of each metal in zebrafish, but did not find any antagonistic interactions of Cd and Zn. This was possibly due to the much lower dose ratio (1:35) of Cd and Zn employed by Arini et al. (2015) in the mixture treatment relative to that (1:25) in the present study.

Metallothionein plays a primary role in maintaining cellular homeostasis and detoxification of metals, especially during chronic exposure (Ahearn 2010). It has also been suggested that the induction of MTs are negatively correlated with cellular energy allocation (Erk et al. 2008). In the current study, we observed a significant upregulation of hepatic expression of the MT gene in fish exposed to waterborne Zn, alone and in combination with Cd, whereas no change in MT expression was recorded in fish exposed to waterborne Cd alone (Fig. 3.1D). A similar trend in MT expression in tissues was also reported previously in fish chronically exposed to waterborne Cd and/or Zn

(Arini et al. 2015, Driessnack et al. 2016). These findings suggest that the changes in hepatic MT expression observed in our study was essentially induced by the increase in Zn tissue burden, as the combined exposure to Cd and Zn did not elicit any additive response on MT expression. Thus, it is logical to suggest that the reproductive impairment in fish exposed to the binary mixture of waterborne Cd and Zn in our study was induced predominantly by the disruption of estrogen-mediated functions, and less likely by the increased metabolic cost of coping with elevated Cd and Zn burden.

3.5. Conclusion

Overall, our study has demonstrated that chronic exposure to waterborne Cd and Zn in binary mixture can elicit a greater than additive effect on FHM reproduction. Fish fecundity was found to be the most sensitive endpoint for assessing the reproductive effect of Cd and Zn in mixture. Although both Cd and Zn in individual exposures disrupted important estrogenic parameters (e.g., circulating estradiol, hepatic expression of Vtg gene), such changes did not affect fish fecundity. Exposure to Cd and Zn in mixture resulted in a further suppression of plasma estradiol and also increased follicular atresia, which corresponded with an almost 50% reduction in fecundity compared to the control. The interaction of Cd and Zn in mixture also resulted in decreased Cd accumulation in tissues (gill and liver). However it did not influence Zn accumulation in FHM tissues. Exposure to Zn, not Cd, increased the hepatic expression of MT gene, and the co-exposure to Cd and Zn did not elicit any additive effect on MT expression. Overall, our findings suggested that the impairment of reproductive output in FHM exposed to waterborne Cd and Zn in mixture occurred predominantly due to the disruption of pathways involved in estrogen synthesis and signaling.

CHAPTER 4:

IMPAIRMENT OF ESTRADIOL PRODUCTION DURING *IN VITRO* INCUBATION OF FATHEAD MINNOW (*PIMEPHALES PROMELAS)* OVARIAN EXPLANTS IN THE PRESENCE OF CD, CU AND ZN, ALONE AND IN BINARY MIXTURE

4 ***Preface***

This chapter used *in vitro* techniques to assess the ability of Cd, Cu and Zn alone and in binary combination (Cd-Cu and Cd-Zn) to affect estradiol production in fathead minnow (FHM) ovarian explants. First, using an increasing range of exposure concentrations, an IC_{50} for estradiol production was determined for each metal. Subsequently, the determined IC_{50} values were used to assess the interactive effects of binary metal mixtures on *in vitro* estradiol production using a toxic unit approach. For assessing the interactive effects of metals in binary mixture, the first metal (Cd, Cu or Zn) was held constant at the IC_{50} while the concentration of the second metal was varied in the exposure (IC_{50}, 0.5 $IC_{50,}$ and 0.25 IC_{50}). Overall, the study indicated each metal impairs estradiol production more or less in a dose-dependent manner, whereas the interactive effects (additive or less than additive) of binary metal mixtures vary depending on the dose ratios of each metal in the exposure.

4.1 Introduction

In vertebrates, steroid hormones play a crucial role in development, homeostasis, immune system function, growth and reproduction (Norris 1997, Ornostay et al. 2016). In particular, the sex steroid hormones, estrogens, and androgens are vital in the regulation of reproduction. Synthesis of sex steroid hormones is primarily performed by the gonads (ovary and testis), via the conversion of cholesterol through a series of biochemical processes catalyzed by cytochrome P450 enzymes (Nagahama 1994). The transport of cholesterol is mediated by the protein StAR, which

is one of the rate-limiting steps in the production of estradiol. Cholesterol is first converted into pregnenolone in theca cells, which is then further modified to produce 17α-hydroxyprogesterone (17α-OHP), and testosterone (T) through a series of P450 mediated reactions. In ovaries, the theca cells release T to be taken up by granulosa cells, where it is converted to 17β-estradiol by cytochrome P450 aromatase (CYP19) (Miller 1988, Nagahama 1994, Villeneuve et al. 2007). In teleost species, such as the FHM the estradiol produced in the ovaries is released into the circulation, and the estradiol then stimulates the liver to produce vitellogenin, a yolk precursor, which plays a vital role in oocyte maturation (Mizuta et al. 2013).

In recent years, there has been an increasing desire to use *in vitro* testing methods in predictive as well as mechanistic ecotoxicology (Villeneuve and Garcia-Reyero 2011, Johnston et al. 2014). The employment of cell lines and organ cultures allow for better control of exposure conditions, and helps to study mechanisms of toxic actions of contaminants without systemic control, and also to reduce the use of animals for toxicity assessments (Eisenbrand et al. 2002, Johnston et al. 2014). The culture of vital organ(s) from model organisms is increasingly being used as a potential screening tool to identify modes of toxic action and link them to adverse outcomes in whole organisms (Eisenbrand et al. 2002, Ankley et al. 2010, Villeneuve and Garcia-Reyero 2011, Johnston et al. 2014). *In vitro* incubation of gonadal tissues has been demonstrated to be a viable alternative method for assessing the effects of contaminants on the metabolic capacity of gonadal tissue and reproductive fitness in fish (McMaster et al. 1995). Ovarian explants have been used to estimate changes in steroidogenesis (estradiol, testosterone production) following exposure to test compounds, specifically, those suspected of being endocrine disrupting compounds (McMaster et al. 1995, Breen et al. 2007, Villeneuve et al. 2007).

Endocrine disrupting chemicals (EDCs) are defined as exogenous agents that interfere with the production, release, transport, metabolism, binding, action or elimination of natural hormones in the body responsible for maintenance of homeostasis and the regulation of developmental processes (Kavlock and Datson 1996, Scholz et al. 2012). Metals such as Cd, Cu and Zn are most commonly observed to cause toxicity in fish via disruption of ion homeostasis, especially during acute exposures (see Wood 2012 for review). However, there is growing evidence in mammalian studies that these metals (Cd, Cu, and Zn) may also act as metalloestrogens, and impair reproductive performance through interference with estrogen receptors and/or by disrupting estrogen-mediated functions (Martin et al. 2003, Darbre 2006). Our previous work has indicated that chronic waterborne exposure to Cd, Cu and Zn, alone and in binary mixtures, may significantly impair the reproductive performance in fish, by affecting a wide range of endpoints (fecundity, circulating estradiol levels, ovarian histopathology, hepatic gene expression of estrogen receptors and vitellogenin) (Driessnack et al. 2016, 2017). With this in view, the present study was designed: (i) to examine the dose dependent effects of Cd, Cu or Zn on the *in vitro* estradiol production in ovarian explants of fish, (ii) to assess the interactive effects (less than additive, additive, or more than additive) of these metals in binary mixtures on *in vitro* estradiol production, and also (iii) to evaluate how these interactive effects are influenced by the dose ratio of these metals in the exposure medium. We used a standardized protocol, as described by McMaster et al. (1995), which uses the incubation of isolated ovarian tissue of FHM with specific toxic agents and subsequent evaluation of ovarian steroid production.

4.2 Materials and methods

The ovarian steroidogenesis *in vitro* assay for FHM employed in this study was adapted from methods standardized by McMaster et al. (1995), with modifications described in Villeneuve

et al. (2006) and Breen et al. (2007). The assay was used to measure: (i) the dose-dependent effects of Cd, Cu or Zn on *in vitro* estradiol production in FHM ovary, and (ii) the interactive effects of Cd-Cu and Cd-Zn mixtures at different dose ratios on *in vitro* estradiol production in FHM ovary. Ovarian tissue was dissected out from sexually mature female FHMs (age 6-9 months), placed in fresh media and carefully divided into 10-20 mg pieces, and the tissue weights were used later to normalize estradiol production. Explants were taken from n = 40 female FHMs, with sections from each female being used across all treatment concentrations assessed. The ovary explants were then added to 48-well cell culture plates (Falcon 35-3078, Beckton Dickinson Franklin Lakes, NJ, USA). Each well in the culture plate contained the supplemented media (495 µl) and corresponding metal treatment solutions (5 ul). Supplemented media was comprised of Media 199 (Hi-Media Laboratories Ltd) containing phenol red, which was supplemented with 0.1mM IBMX (3-isobutyl-1-methylxanthene, Alfa Aeser) and 1 ug/L of 25-hydroxycholesterol (Sigma-Aldrich). Stock solutions of Cd, Cu, and Zn, were made using the $CdCl_2 \cdot 2.5H_20$, $CuCl_2 \cdot 5H_20$, $ZnCl_2$ (Sigma-Aldrich), respectively, which were then diluted in supplemented media, as required. All plates containing ovary explants were incubated at 18 °C for 24 hrs. Following incubation, media was removed from each well, flash frozen in liquid nitrogen, and then stored at -80 °C until analyzed for estradiol level. Estradiol was measured using commercially available ELISA kits obtained from Cayman Chemicals (Cayman Chemical, MI, USA). Media samples were analyzed in duplicate at two different dilutions following manufacturers' protocols, and were quantified using a Varioskan Flash multi-mode plate reader (Thermo Fisher Scientific, ON, Canada) at 405-420 nm wavelength.

For assessing the effects of individual metals, Cd, Cu or Zn was tested at six different concentrations with six replicate ovary explants per concentration. Each plate also contained six replicate control ovary explants, from which subsets of control samples were randomly selected

for estradiol determination (n = 4). The nominal concentrations tested for each metal were: Cd at 1, 5, 10, 20, 30, and 70 μM; Cu at 1, 5, 10, 40, 75, 150 and 300 μM; and Zn at 2.5, 5, 10, 40, 75, and 250 μM. Upon completion of the *in vitro* single metal exposures, media estradiol concentrations were used to determine the IC_{50} for each metal, based on the inhibition of estradiol production relative to control. Values for IC_{50} were generated with SigmaPlot 11.0 (Systat Software Inc, San Jose, California, USA) and using a four-parameter logistic fit function. Statistical differences between treatment concentrations were assessed using a One-Way Analysis of Variance (1-Way ANOVA). If the assumptions of the 1-Way ANOVA (independent samples, normality and equal variance of the data) were met and a significant response ($p \leq 0.05$) was noted, the Dunnett post hoc test was used to denoted any difference from the control. Failure to meet the 1-way ANOVA assumptions, even after arcsin transformation, resulted in the use of the non-parametric Kruskal-Wallis test, with Mann-Whitney test used as a post hoc assessment.

The evaluation of binary metal exposures on *in vitro* estradiol production in FHM ovary was done with either Cu, Cd or Zn held constant at the calculated IC_{50} level, while the competing metal was varied at 100%, 50% or 25% of IC_{50} level, which allowed assessing the metal mixture toxicity using a toxic unit approach (Sprague 1970, Norwood et al. 2003). Combinations of metals tested were: Cd (IC_{50}) with Zn or Cu as the variable, Zn (IC_{50}) with Cd as the variable, and finally Cu (IC_{50}) with Cd as the variable. Statistical analysis of mean % inhibition of *in vitro* ovarian estradiol production by the metal mixture treatment relative to the control were assessed using a t-test with Bonferroni correction for multiple comparisons, when the data met the assumptions of normality and equal variance. In situations when the data did not meet these assumptions, the Mann-Whitney test with Bonferroni correction was employed for assessing statistical difference.

4.3 Results and discussion

The goal of this study was to assess the feasibility of *in vitro* assays for studying the effects of metals directly on ovarian steroidogenesis, alone or in binary mixtures. To the best of our knowledge, no previous studies have used an *in vitro* experimental approach to assess the effects of metals on steroidogenesis in fish. In general, the present study demonstrated that exposures to metals, individually as well as in binary mixtures, alter the production of estradiol in FHM ovary explants. Also, the interactive effects of metal mixtures were strongly influenced by the dose ratio of metals in the mixture.

4.3.1 Effects of individual metals on *in vitro* steroidogenesis

For each of the metals (Cd, Cu, and Zn) tested, an IC_{50} for estradiol production was derived using the measured estradiol level in the media following exposure to an increasing range of concentrations. In general, a dose-dependent decrease in *in vitro* estradiol production was observed with each metal (Fig. 4.1). The four-parameter logistic fit analysis revealed IC_{50} values for Cd (18.0 μM, 95% CI 12.77 – 87.23), Cu (3.3μM, (95% CI 26.88 – 73.12), and Zn (2.8 μM, 95% CI 29.37 – 73.12).

Statistical results of the Cd estradiol data noted only a statistically significant difference for the highest concentration (70 μM) compared to control, despite marked decreases in production at the higher dosing concentrations (Fig. 4.1A). This is likely a result of the smaller sample size (n = 4) that was used. Due to limited mature ovarian explants availability, the variability in the samples was a bit greater than would be preferred. The Cd data set was assessed using a series of t-tests, using the same assumptions for parametric testing as detailed in the methods. This alternative option was used to account for the variability and upregulation in the data set and to

better visualize changes in the data. Both Cu and Zn demonstrated inhibition compared to control across the concentration ranges tested (Fig. 4.1 B, C). Statistically significant decreases in estradiol production were noted for the higher concentration ovarian explant replicates in the Cu and Zn treatments.

Among the three metals examined in this study, the effects of Cd on steroidogenesis have probably been studied more extensively than the effects of other metals. Mammalian studies with human granulosa cells and rat ovaries have demonstrated that Cd can impair steroidogenesis through decreased progesterone production and expression of StAR and P450scc (side chain cleavage) (Piasek et al. 2002, Zhang and Jia 2007, Zhang et al. 2008). Recent studies have also suggested that Cd-induced reproductive impairment in fish occurs, at least in part, due to the attenuation of steroid production at the hypothalamus, pituitary and even gonadal levels (Tilton et al. 2003, Vetillard and Bailhache 2005, Das and Mukherjee 2013, Driessnack et al. 2016). Interesting, however, in the present study *in vitro* ovarian estradiol production was found to be much less sensitive to Cd in comparison to Cu or Zn, which was not consistent with the findings of previous *in vitro* studies in fish (Olabarrieta et al. 2001, Tan et al. 2008). For example, Tan et al. (2008) reported that the ovary cell line of channel catfish (*Ictalurus punctatus*) was more sensitive to Cd than Zn, although a different set of endpoints (cell cytotoxicity, proliferation, and morphology) was assessed in that study. Nonetheless, our observations are in agreement with the findings of Das and Mukherjee (2013), who also assessed the effect of Cd on the secretion of estradiol by the ovarian follicles of common carp (*Ctenopharyngodon cyprini*). They demonstrated that Cd was able to impair estradiol production in ovarian tissues, and also suggested that the reduction in estradiol was linked with Cd-induced impairment of LH (luteinizing hormone) and P450-aromatase expression and activity. In addition, they found that SF-1 (steroidogenic factor),

which activates the aromatase enzyme, was affected as well. In their study, Das and Mukherjee (2013) indicated that 2.0 μM Cd was a physiologically "safe" dose for ovarian steroidogenesis, which is consistent with the findings of the present study. It is important to note here that chronic exposure to waterborne Cd (0.06-0.09 μM) has also been found to decrease the circulating estradiol level in female fish (Tilton et al. 2003, Driessnack et al. 2017). At present, there is no previous evidence of a Cu-induced decrease in *in vitro* ovarian estradiol production either in fish or in mammalian systems. Zhang et al. (2016) reported reduced circulating FSH, LH, estradiol and progesterone levels as well as downregulation of genes involved with steroidogenesis in yellow catfish (*Pelteobagrus fulvidraco*) chronically exposed to waterborne Cu (0.5-1.0 μM), although no decrease in circulating estradiol level was recorded in female FHM exposed to similar concentration of waterborne Cu (Driesnack et al. 2016). Mammalian evidence also suggests that Cu can impair the production of androstenedione in theca cells, an intermediate in the production of estradiol from cholesterol (Kendall et al. 2006).

As observed with Cu, *in vitro* FHM ovarian estradiol production was also found to be equally sensitive to Zn (similar IC_{50} values for both metals). In line with this observation, our previous study also demonstrated that chronic exposure to waterborne Zn (2.6 μM) reduces circulating estradiol level in female FHM (Driessnack et al. 2017). A mammalian study assessing the effects of dietary Zn exposure in male rats has noted a decrease in hepatic conversion of testosterone to dihydrotestosterone, and reductions in circulating estradiol, testosterone and LH levels (Om and Chung, 1996). Overall, it seems that the observed decreases in *in vitro* estradiol production in FHM ovary following short-term exposure to Cd, Cu or Zn were mediated by their effects on the ovarian steroidogenesis and/or histopathology.

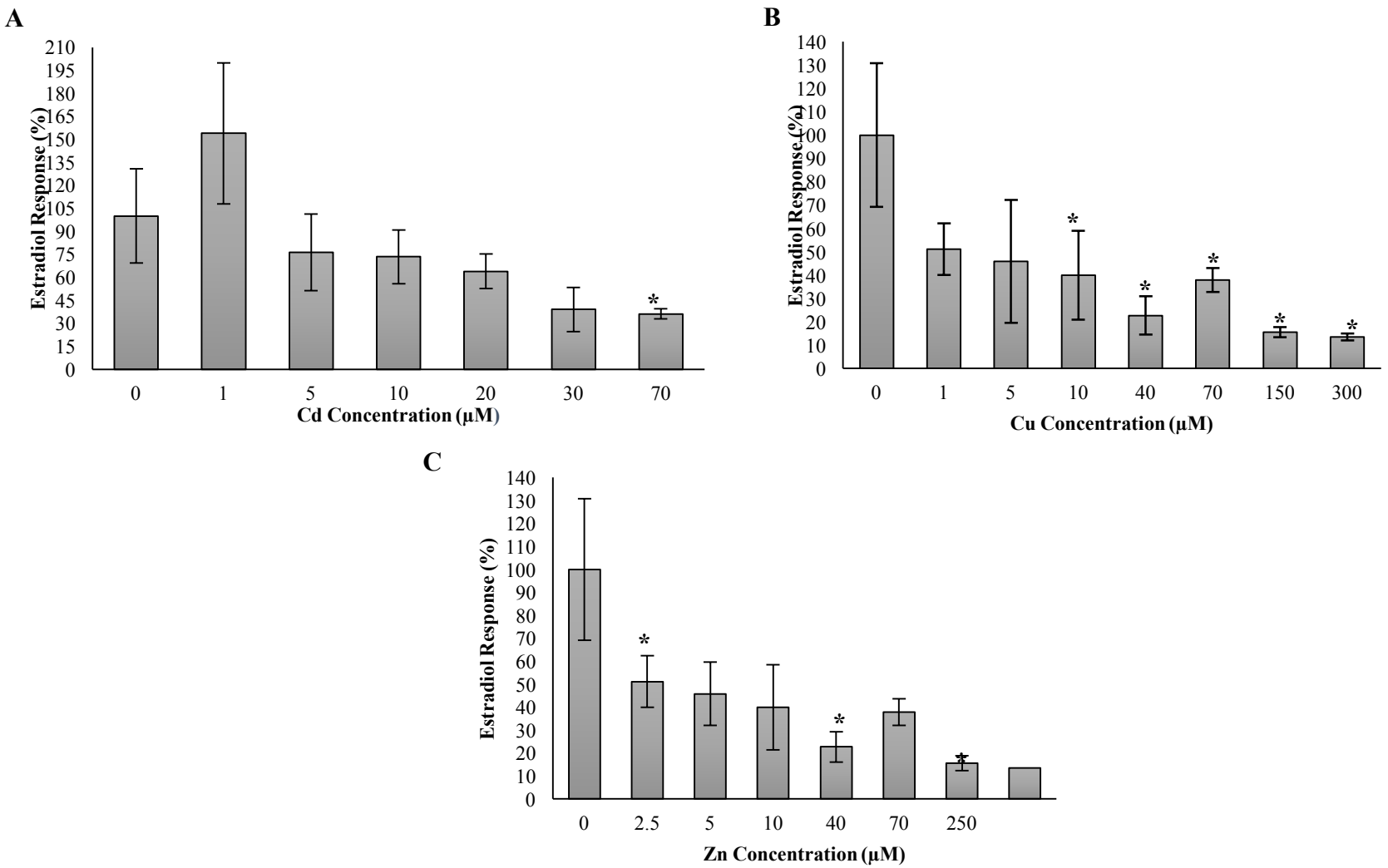

Figure 4.1: Mean percent (inhibition) of estradiol production in fathead minnow ovarian explants relative to control following exposure to either Cd, Cu or Zn over a series of concentrations (0 – 250 µM). Data are shown as mean ± SEM (n= 4). All metals were assessed using the Kruskal-Wallis test with the Mann-Whitney test for post hoc determination. Statistical differences ($p \leq 0.05$) from the control were denoted with - *.

4.3.2 Effects of binary metal mixtures on *in vitro* steroidogenesis

The assessment of effects of metal mixtures at different dose ratios was performed using the toxic units (TU) approach (Sprague 1970, Norwood et al. 2003). Based on this approach, the IC_{50} for each metal was considered to be 1 TU. Therefore the predicted response were derived by the addition of the TU present in the mixture. For example, when both metals were present at their respective IC_{50} values in the mixture (i.e., 1 TU of Metal A plus 1 TU of Metal B), a complete (100%) inhibition of *in vitro* ovarian estradiol production was predicted. Similarly, we assumed that when the mixture contained one metal at its IC_{50} and the other metal at its 0.5 or 0.25 IC_{50}, it would result in 75% or 62.5% inhibition of estradiol production The predicted and observed percentage inhibitions of *in vitro* ovarian estradiol production, induced by binary metal mixture treatments in comparison to the control, were presented in Table 4.1. In general, results of this *in vitro* study indicated that when the metal mixture exposure contained one metal at 1 TU and the other metal either at 1 or 0.5 TU, a less than additive inhibition of estradiol production occurred. On the other hand, an additive or more than additive inhibition of estradiol production was typically observed when the mixture contained one metal at 1 TU and the other metal at its lowest tested dose (0.25 TU).

In the present study, the observed less than additive effects of metal mixtures, when both metals were present at higher toxicity dose ratios (e.g., each at 1 TU), occurred possibly due to their competitive interaction for uptake in ovarian tissues. Waterborne Cd has been reported to reduce short-term (3hr) branchial Zn uptake and vice versa in fish, but this competitive effect was mainly evident when both metals were present at their respective 96 hr LC_{50} levels (Niyogi et al., 2015). Similarly, waterborne Cu has also been found to reduce branchial Cd uptake and vice versa in fish, when both of these metals were present at their 96 hr LC_{50} levels in the exposure (Niyogi

Table 4.1: Predicted (based on toxic unit approach) and observed percentage inhibition (relative to the control) of estradiol production in isolated FHM ovary in primary culture following exposure to the different dose ratios of binary metal mixtures (Cd vs. Cu, and Cd vs. Zn). One toxic unit of each metal was equivalent to its IC_{50} for estradiol production. Statistically significant differences ($p \leq 0.05$) in comparison to the control are indicated by the asterisk (*).

Treatment	% Inhibition Predicted	% Inhibition Observed	Interaction
Cd IC50 + Cu IC50	100 (-)	76.9 (-) *	Less than Additive
Cd IC50 + Cu 0.5 IC50	75 (-)	52 (-)	Less than Additive
Cd IC50 + Cu 0.25 IC50	62.5 (-)	76.5 (-) *	More than additive
Cu IC50 + Cd IC50	100 (-)	76.9 (-) *	Less than Additive
Cu IC50 + Cd 0.5 IC50	75 (-)	47.7 (-)	Less than Additive
Cu IC50 + Cd 0.25 IC50	62.5 (-)	66.8 (-) *	Additive
Cd IC50 + Zn IC50	100 (-)	63.7 (-) *	Less than additive
Cd IC50 + Zn 0.5 IC50	75 (-)	9.6 (-)	Antagonistic
Cd IC50 + Zn 0.25 IC50	62.5 (-)	38.1 (-) *	Antagonistic
Zn IC50+ Cd IC50	100 (-)	63.7 (-) *	Less than additive
Zn IC50+ Cd 0.5 IC50	75 (-)	32.1 (+)	Antagonistic
Zn IC50 + Cd 0.25 IC50	62.5 (-)	63 .5 (-) *	Additive

et al., 2015). On the other hand, we generally observed either additive or more than additive effect, when one of the metals in the binary mixture was present at a much lower toxicity dose (0.25 TU) relative to that of the other (1 TU), which was consistent with the findings of our *in vivo* reproductive bioassays in FHM. For example, we previously reported a greater than additive effect on circulating estradiol level in female FHM during chronic exposure to lower toxicity dose levels (~10% of 96h LC_{50}) of waterborne Cd and Cu (Driessnack et al., 2016). Similarly, we also recorded an additive effect on circulating estradiol level in female FHM during chronic exposure to the mixture of waterborne Cd and Zn, each present at their ~10% of 96h LC_{50} (Driessnack et al., 2017). Consistent with our findings, Amorim et al. (2011) reported that Cd and Zn elicited a less than additive effect on the growth of springtails (*Folsomia candida*), when both metals were present at their respective EC_{50} levels in the mixture, but induced a greater than additive effect when present in lower toxicity dose ratios (e.g., EC_{10}). Moreover, Gennings et al. (2002) observed a similar trend (synergism at low doses and antagonism at high doses) in cytotoxicity of human epidermal keratinocytes during exposure to metal mixtures.

4.4 Conclusion

Overall, the findings of the present study demonstrate that the interactive effects of these metals (Cd, Cu, and Zn) on the steroidogenesis in FHM ovary are complex and strongly influenced by the dose ratios of metals present in the mixture. Although the mechanisms underlying these complex interactions remain unclear, our study provides first direct evidence that Cd, Cu, and Zn, alone as well as in binary mixture, may impair the synthesis and secretion of estradiol from the ovary of female fish. Thus, the *in vitro* observations in the present study further corroborate our previous *in vivo* findings, suggesting that the interactive effects of Cd and Cu or Zn on FHM reproduction occurs due to the disruption of estrogen-mediated pathways.

CHAPTER 5:

EFFECTS OF CHRONIC EXPOSURE TO WATERBORNE COPPER AND NICKEL IN BINARY MIXTURE ON TISSUE-SPECIFIC METAL ACCUMULATION AND REPRODUCTION IN FATHEAD MINNOW (*PIMEPHALES PROMELAS*)

5 Preface

This chapter focused on the interactive reproductive and physiological effects of Cu and Ni in the fathead minnow (FHM). These metals have apparently different modes of toxic action, as Cu is known to compete with sodium ion for branchial uptake and disrupt sodium homeostasis, and Ni, on the other hand, is a respiratory toxicant and suspected to interfere with magnesium homeostasis in fish. Mature breeding trios of FHM were exposed to waterborne Cu-only, Ni-only and combined Cu-Ni for 21-days to assess changes in reproductive performance, tissue specific metal accumulation patterns and hepatic gene expression (estrogen receptors α and β, vitellogenin and metallothionein). Single metal responses indicated that both Cu and Ni individually impair FHM reproductive capacity. The reproductive bioassay also indicated that the co-exposure of Cu and Ni elicits an additive effect on fish fecundity. The findings also suggested that co-exposure to Cu and Ni decreased fish reproductive output by altering energy allocation as well as impairing the oocyte maturation process in the ovary.

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5.1 Introduction

Human activities are the key driving factors for increasing metal concentrations in natural waters, impairing the health of resident organisms, including fish. Organisms inhabiting metal-

contaminated aquatic ecosystems are almost always exposed to multiple metals in mixture (Borgmann et al., 2008; Vijver et al., 2011). However our present understanding of the detrimental effects of metal exposure mainly comes from studies conducted with individual metals. The interactions of metals in mixture can influence their uptake and internal handling, which may eventually ameliorate or amplify the overall toxicity in exposed organisms (Daka and Hawkins, 2006; Borgmann et al., 2008). To date, most of the studies that evaluated the toxicity of metal mixtures in fish have focused on characterizing effects under acute exposure conditions (Balisteri and Mebane, 2014; Clemow and Wilkie, 2015; Niyogi et al., 2015; Brix et al., 2016), and the chronic toxicity of metal mixtures has been studied sporadically.

Copper (Cu) and nickel (Ni) are metals, which exhibit a high degree of shared natural and anthropogenic release into the aquatic ecosystems (ASTDR, 2004; Charles et al., 2014). Although Cu is an essential metal to fish, it can cause toxicity when its concentration exceeds the physiological threshold in the body (Grosell, 2012). Short-term waterborne exposure to high concentrations of Cu elicits toxicity in fish primarily by impairing sodium (Na^+) and chloride (Cl^-) homeostasis (Grosell and Wood, 2002; Morgan et al., 2004). On the other hand, long-term chronic exposure to sub-lethal concentrations of waterborne Cu leads to increased Cu accumulation in vital body tissues (gill, liver and kidney) of fish (Kamunde et al., 2005; Niyogi et al., 2006; Driessnack et al., 2016), resulting in various adverse physiological consequences. Chronic Cu exposure to fish has been linked with reduced growth and survival (Hansen et al., 2002; Niyogi et al., 2006), impaired essential ion balance in the plasma (Na^+, Cl^-, and Cu^+) (Niyogi et al., 2006), altered nitrogen excretion (De Boeck et al., 1995), decreased swimming capacity (Campbell et al., 2002), oxidative damage (Eyckmans et al., 2011), and diminished stress response (Gagnon et al., 2006). Moreover, long-term exposure to relatively low levels of waterborne Cu

(e.g., 10-fold lower than its 96h LC_{50}) has been reported to decrease the reproductive output (egg production) in fish (McKim and Benoit, 1971; Horning and Neiheisel, 1979; Driessnack et al., 2016). Our recent work indicated that this might occur as an indirect effect of Cu-induced alteration of energy allocation in fish (Driessnack et al., 2016).

Unlike Cu, the essentiality of Ni in fish remains unsubstantiated (Chowdhury et al., 2008; Pyle and Couture, 2012). Waterborne Ni during acute exposure has been found to act primarily as a respiratory toxicant rather than an inonoregulatory toxicant like Cu (Pane et al., 2003; 2004a). It has also been reported that acute exposure to waterborne Ni interferes with magnesium (Mg^{2+}) reabsorption in the kidney, leading to increased loss of Mg^{2+} through urine and thereby decreasing plasma Mg^{2+} levels (Pane et al., 2005). In contrast, the effects of chronic exposure to Ni are not well documented in fish. It has been reported that waterborne Ni during chronic exposure induces severe morphological and histopathological damage to vital organs in fish, mainly in the gill, liver, and kidney (Nath and Kumar 1989; Athikesavan et al., 2006; Pane et al., 2004). Chronic exposure to waterborne Ni also leads to increased accumulation of Ni in target organs (Ptashynski and Klaverkamp 2002; Alsop et al., 2014), and in particular histopathological damage associated with increased Ni accumulation in the gill is known to cause significant reduction in gill diffusion capacity and impair respiratory function and swim performance in fish (Hughes et al., 1979; Pane et al., 2004b). In addition, chronic exposure to both waterborne and dietary Ni also causes reproductive toxicity in fish. A significant reduction in egg production and/or egg hatchability has been reported in fish following chronic exposure to Ni (Pickering, 1974; Dave and Xiu 1991; Alsop et al., 2014). However the physiological mechanisms underlying such effects are unknown.

Our recent studies indicated that the chronic interactions of metals with apparently different mode of toxic action (e.g., Cd (a calcium antagonist) and Cu (a sodium antagonist); Niyogi and

Wood, 2004) can elicit greater than additive effects on the reproductive output in fish (Driessnack et al., 2016). Since reproductive capacity in fish has been suggested to be sensitive to chronic waterborne exposure to Cu or Ni (Grosell, 2012; Pyle and Couture, 2012), the present study was designed to evaluate their interactive effects on the reproductive performance in fish. In addition, we also aimed to gain insights into the potential physiological mechanisms underlying their interactive effects. We exposed sexually mature FHM to ~10% of 96-h LC_{50} for waterborne Cu and Ni, singly and in mixture, in addition to the control (no metals added). The specific objectives of this study were: (i) to examine the interactive effects of waterborne Cu and Ni on fish reproductive performance, (ii) to evaluate how Cu and/or Ni exposure affect the female hepatic expression of genes that play important roles in regulating reproduction and metal-detoxification (estrogen receptors (ER-α and ER-β), vitellogenin (Vtg), and metallothionein (MT)), (iii) to evaluate how exposure to Cu and/or Ni affect the female plasma sex steroid (estradiol) level, and (iv) to examine whether the interactions of Cu and Ni alter the tissue-specific (gill, liver, ovary, and carcass) accumulation of each metal. Our assumption was that the chronic interactions of waterborne Cu and Ni would not influence their tissue-specific accumulation profile, and induce additive or greater than additive effect on FHM reproductive performance.

5.2. Material and methods

5.2.1. Experimental design and setup

We conducted the present study in the Aquatic Toxicology Research Facility (ATRF) of the University of Saskatchewan. The experimental protocol employed in this study was approved by the University of Saskatchewan Animal Care Committee and met Canadian Council of Animal Care protocols. Adult FHMs (8-9 months old) used in this study were taken from the in-house

cultures, and females were approximately 1 g in weight and 3.5-4 centimeters (cm) in length, and males were roughly 2.5 g in weight and 5-5.5 cm in length. Fish were reared in dechlorinated Saskatoon city water (Ca^{2+} 44, Mg^{2+} 18, Na^{+} 26, K^{+} 3, Cl^{-1} 11, SO_4^{-2} 50, hardness 155, alkalinity 110 (both as $CaCO_3$), dissolved organic carbon (DOC) 2.5 (all in mg/L), pH 7.9). The methodology used to analyze the water quality parameters are presented elsewhere (Driessnack et al., 2016). A fully factorial 2-way experimental design was employed to examine the effects of waterborne Cu and Ni, both individually and in mixture. The experiment was comprised of the following four treatments: (i) control (dechlorinated Saskatoon municipal water with no added metals), (ii) waterborne Cu (50 µg/L, added as $CuCl_2$ (Sigma-Aldrich, MO, USA)), (iii) waterborne Ni (275 µg/L, added as $Ni(NO_3)_2{\cdot}6H_2O$ (Sigma-Aldrich, MO, USA)), and (iv) Cu and Ni in mixture (50 µg/L and 275 µg/L, respectively). Waterborne Cu and Ni concentrations reported here are nominal concentrations and correspond to ~10% of 96-h LC_{50} for Cu and Ni, respectively, for FHM under ambient water chemistry (unpublished data). The measured values for Cu and Ni in the exposure water are reported in Table 1.

Fish reproductive bioassay was performed based on a standardized methodology (Ankley et al. 2001), and has been described previously in Driessnack et al. (2016). Briefly, an enclosed proportional diluter system was set up using 21 individual 9-L glass aquaria (five replicates for each metal(s) treatment, and six replicates for control). Each aquarium contained one polyvinyl chloride breeding tile and one FHM breeding trio (1 male: 2 female). Exposure waters were prepared using a proportional dual head metering pump (Q2V, Fluid Metering Inc., NY, USA) drawing at a fixed rate from a control water head tank and the corresponding metal treatment head tank. A pressurized manifold system (JCM Specialties, SK, Canada) was used to ensure mixing of water coming from both head tanks, and the appropriate exposure water was then supplied to the

corresponding aquaria. The metering pumps were set to draw at a rate of 3 turnovers per day for each aquarium. All aquaria were kept in a temperature-controlled (25±2°C) water bath, and a 16h:8h light and dark cycle was maintained.

5.2.1.1. Pre-exposure period

A 21-day pre-exposure phase was performed to ensure that the FHM trios selected for use in the exposure demonstrated an established and comparable level of reproductive output. Fish (n = 40 trios) were selected at random from the in-house culture and assessed for fork length, weight, and secondary sex characteristics before placement in the 9-L aquaria. Breeding trios were fed a commercial frozen bloodworm diet (Sally's BloodwormsTM, San Francisco Bay Brand Inc., Newark, CA, USA) twice daily at a ration of approximately 10-12% of combined body weight (~1g/trio). All uneaten food and feces were removed from the aquaria approximately 10-15 minutes post-feeding, and all tanks were cleaned every other day during both pre-exposure and exposure periods. Egg production was assessed daily by removal and examination of spawning tiles for deposition of eggs using methods described previously in Driessnack et al. (2016). FHM trios chosen for the exposure phase demonstrated 100% survival of adults, > 80% egg fertilization, and successful spawns every 2-3 days, and produced 10-20 eggs/female/day (OECD 2003; Driessnack et al., 2016). Of the 40 FHM trios used in the pre-exposure phase, 22 met the pre-set criteria mentioned above, and we selected 21 trios with the greatest mean egg production for the exposure phase. A one-way analysis of variance (ANOVA) was applied to examine the statistically significant differences among the 21 selected FHM trios in mean eggs/trio, eggs/female/day, fertilization success, and a Chi-Square test was used to compare the number of spawning events. No statistically significant differences were found in any of the reproductive parameters described above ($p \leq 0.05$).

5.2.1.2. Exposure phase

Exposure to metal(s) commenced immediately following pre-exposure and continued for 21-days. Spawning tiles were evaluated daily for deposition of eggs as described in the pre-exposure phase. Photographs of each brood were used to determine the number of eggs. Mean eggs per FHM trio per day in each treatment were estimated by dividing the total eggs produced over 21-days by the number of FHM trios used and exposure period. For each brood, ten eggs were randomly selected for the estimation of egg size (as the diameter (mm)) using Image J software (imagej.nih.gov). Collected eggs were assessed for fertilization success, hatching success, time to hatch, 5-day post hatch larval survival and deformity prevalence (Driessnack et al., 2016). All eggs and larvae were held in aerated control water. The larvae were maintained for eight weeks post-hatch for assessing the differences in morphometrics (length and weight). Water samples (2-mL) were collected daily from an aquarium selected randomly from each treatment for the determination of total metal levels. At the end of the exposure phase, fish were euthanized with AquaCalm (Syndel, BC, Canada) assessed for fork length (mm), total body weight (g), and secondary sex characteristics (nuptial tubercles, body banding, and fin dot). Blood was collected from female fish by caudal vein puncture using heparinized needles for the estimation of circulating estradiol level. For the measurement of tissue-specific metal accumulation, female fish were dissected out, and the gills, liver, ovary, and carcass were carefully removed, rinsed in deionized water, blotted dry and transferred into the pre-weighed polythene vials. The vials were re-weighed to record the tissue weight to the nearest 0.01 mg. A portion of the ovary was preserved separately for the analysis of ovarian histopathology. In addition, the liver from the other female in each trio was dissected out and placed immediately in RNAlater (Life Technologies, ON, Canada), which was subsequently used for the gene expression analysis.

5.2.2. Experimental analysis

The exposure water in each treatment was measured daily for conductivity, dissolved oxygen, ammonia, and temperature as described in Driessnack et al. (2016). Water samples collected during the exposure phase for dissolved metal analysis were passed through a 0.45 μm syringe filter, acidified (0.2% HNO_3, trace metal grade, VWR, ON, Canada) and stored at 4°C until analyzed. All of the fish tissues were digested in 5 volumes of 1N HNO_3 at 60°C over 48 h, and then stored at 4°C until analyzed for metal concentrations. Digested tissues and water samples were analyzed for Cu and Ni concentrations in a graphite furnace atomic absorption spectrometer (AAnalyst 800, Perkin-Elmer, CT, USA). To maintain the quality control and assurance of metal analysis, appropriate method blanks and certified standards for Cu and Ni (Perkin-Elmer, CA, USA) were used. Furthermore, to validate the measurement of Cu and Ni concentrations in fish tissue samples, a certified reference material (TORT-3; National Research Council, Canada) was also analyzed using the same procedure described above. A recovery of 106% and 91% for Cu and Ni, respectively, was recorded in the reference material.

For the analysis of circulating estradiol level, female blood samples were centrifuged at 2000g for 10 minutes immediately after collection. The separated plasma was then collected and stored at -80 ^{0}C until analysis. Plasma estradiol levels were determined using a commercial ELISA Kit (Cayman Chemicals MI, USA), following the manufacturer's instructions. Ovaries collected for histological analysis were placed in biopsy cassettes, fixed in 4% paraformaldehyde (PFA) for 5 hrs, and then transferred to 70% ethanol until processing. The ovarian tissues were processed following the method described in Driessnack et al. (2017). Briefly, tissues were dehydrated through a series of graded ethanols (70-100%) and perfused with paraffin wax, as recommended in established protocols (US EPA, 2006; OECD, 2009), and then fixed in paraffin. The paraffin

blocks were then sectioned into 5-7 μm thick sections using a rotary microtome (HM330; Heidelberg, Germany), and the sections were fixed on pre-cleaned suprafrost slides (VWR, ON, Canada), de-paraffinized and stained using Meyer's hematoxylin and eosin (US EPA, 2006; OECD, 2009). Following staining, slides were examined under a Zeiss Axioplan Fluorescence Microscope and photographed using an AxioCamICc1 (Colour, 1.4 MP) digital camera. Sections of ovary were photographed in 2-3 non-overlapping areas for follicular staging and counting. Since the distribution of oocytes within the ovary is random, each section was considered representative of the whole ovary (n = 15-20 females FHMs) (Wolf et al., 2004). Examination of FHM ovarian sections was performed using Image J (imagej.nih.gov). For each ovarian section, approximately 50 follicles were counted and determined to be either: previtellogenic (primary oocytes, cortical alveolar), post-vitellogenic (early or late vitellogenic, mature), or atretic (underdeveloped or degenerating). Different types of follicles were estimated as a proportion of total follicles (e.g., number of previtellogenic follicles per total follicles counted), as described previously by Wolf et al. (2004) and Leino et al. (2005).

For gene expression evaluation, whole female liver samples preserved in RNALater were removed from the solution, homogenized using a handheld tissue homogenizer, and RNA extracted using an illustra RNAspin Mini Kit (GE Healthcare, ON, Canada). The methodology employed for the purification of RNA and subsequent synthesis of cDNA has been described elsewhere (Driessnack et al., 2016). All of the primers used for the gene expression analysis were synthesized by Integrated DNA Technologies (IDT, IA, USA), following gene primer sequences (ER-α, ER-β, Vtg, MT and β-actin (used as a housekeeping gene)) reported previously by Werner et al. (2010). Before initiation of RT-PCR (real time quantitative polymerase chain reaction), each gene primer was first optimized to determine ideal primer and $MgCl_2$ concentrations for each gene.

Once optimized, the standard curve was prepared in duplicate to determine linearity, detection range and efficiency for each gene. Optimization and standard curves were run using pooled control female liver cDNA samples (n = 4) in triplicate, including a negative control, and over a 10-fold serial dilution series for the standard curves. Subsequently, RT-PCRs was carried out using an ABI 7300 Real-Time PCR System (Applied Biosystems, Life Technologies, ON, Canada) and a SYBR® Green PCR Master Mix (Applied Biosystems, ON, Canada), following the protocol provided by the manufacturer. The details of the RT-PCR assay conditions have been described previously in Driessnack et al. (2016). Relative expression of each gene was normalized to the housekeeping gene (β-Actin) and evaluated using the 2-ΔΔCT approach, as detailed by Schmittgen and Livak (2008). The changes in the gene expression levels are presented as the average change in transcript abundance per treatment.

5.2.3. Statistical analysis

All statistical tests, were performed using the statistical program SPSS 22.0 (SPSS, Chicago, Il, USA). At first, all data were evaluated for normality (Kolmogorov-Smirnov (K-S) test) and homogeneity of variance (Levene's test) to determine if parametric or non-parametric tests should be employed. Data that failed to meet the assumptions of normality or equal variance were first transformed ($\log_{10}$ +1 or arcsin for percent data) and re-assessed. If log transformation of the data still did not meet the assumptions for parametric testing, a non-parametric extension of the 2-way ANOVA, the Scheirer-Ray-Hare test was performed. Endpoints analyzed using the 2-way ANOVA or the non-parametric analog, Scheirer-Ray-Hare, included all the reproductive parameters except cumulative egg production and cumulative spawning events, gene expression, plasma estradiol concentrations, and Cu and Ni concentrations in the fish tissues and exposure waters. The Kolmogorov-Smirnov (K-S) test was used to compare the differences in cumulative

spawning events and egg production over 21 days between the control and treatments with metal(s). Data for secondary sex characteristics and total spawning events per treatment were assessed using the Chi-Square test.

5.3 Results

5.3.1. Metal exposure concentrations and water quality

The measured concentrations of dissolved Cu and Ni in the experimental exposures have been provided in Table 5.1. The measured concentrations of Cu and Ni in the Cu alone and Ni alone exposures were similar (less than 10% variation) to the target (nominal) concentrations of Cu (50 µg/L) and Ni (275 µg/L), respectively, and were significantly different from that in the control treatment. In contrast, the concentrations of Cu and Ni in the mixture exposure were not significantly different from their concentrations in the Cu alone and Ni alone exposures, respectively. Water quality (conductivity, dissolved oxygen, temperature, ammonia, and pH) was not found to be significantly different among the treatments during the entire duration of the exposure. General water quality parameters are presented in Table A1 of the Appendix.

5.3.2. Fish morphometrics and reproductive performance

We did not record any fish mortality in any treatments over a 21-day exposure period. No significant differences were noted in fish weight (male and female) or secondary sex characteristics among the different experimental treatments (data not shown). In addition, there was no difference in hepatosomatic index (HSI) or gonadosomatic index (GSI) of female FHM across the four different treatments (Table 5.2).

Cumulative egg production in FHM decreased significantly over 21 days of exposure to Cu-only (75% decrease), Ni-only (57% decrease), and Cu and Ni mixture (86% decrease)

treatments relative to the control (Figure 5.1; Table 5.2). Similarly, mean eggs/trio was also significantly decreased following exposure to Cu and Ni, individually and in mixture, relative to the control (Table 5.2). On the other hand, spawning events/trio were significantly reduced following exposure to Cu, alone and in combination with Ni, but not by exposure to Ni alone (Table 5.2). Fertilization success, egg size, and time to hatch (days) were not affected by any of the experimental treatments (Table 5.2). Similarly, no significant differences in hatching success, 5-day post-hatch larval survival and deformities (%), as well as the length or weight of larvae raised for 8-weeks post hatch were recorded among the four experimental treatments (data not shown).

5.3.3. Hepatic gene expression

Female FHM livers were examined for the expression of ER-α, ER-β, Vtg and MT genes. The mRNA expression of ER-α was reduced significantly in fish exposed to waterborne Cu alone relative to the control, although it was not affected by exposure to waterborne Ni, alone or in combination with Cu (Figure 5.2A). In contrast, no significant alterations in the expression of ER-β gene were recorded across the four different experimental treatments (Figure 5.2B). The expression of the Vtg gene did not change significantly following exposure to Cu alone relative to the control, but was significantly upregulated following exposure to Ni alone. In contrast, the Vtg expression was found to be significantly downregulated following exposure to Cu and Ni in mixture relative to the control (Figure 5.2C). The expression of MT gene was significantly increased following exposure to Cu and Ni, individually and in mixture, relative to the control (Figure 5.2D). Moreover, the MT expression in the mixture treatment was significantly higher from that in the Ni only treatment, but not from that in the Cu only treatment.

Table 5.1: Measured dissolved metal concentrations of Cu and Ni in the four different treatment waters. Values are presented as mean ± SEM (n = 21). Significant differences in Cu and Ni concentrations relative to control are indicated by different letters.

Endpoint	Copper	Nickel
Control	2.3 ± 0.6^{A}	0.7 ± 0.2^{a}
Cu-Only	45.6 ± 3.0^{B}	0.5 ± 0.3^{a}
Ni-Only	3.3 ± 0.9^{A}	260.9 ± 16.9^{b}
Cu-Ni	48.0 ± 4.5^{B}	285.4 ± 8.0^{b}

Table 5.2: Reproductive performance and morphometrics in adult female fathead minnows (FHMs) following 21-day exposure to control, Cu-only, Ni-only, and Cu-Ni exposure treatments. Data are presented as mean ± SEM (n= 5-6 trios). Different letters denote statistically significant differences among the four treatments ($p \leq 0.05$). All endpoints assessed were using a 2-Way Analysis of Variance, except the cumulative total eggs data (Kolmogorov-Smirnov (K-S) test; $p<0.008$ (Bonferroni Correction)).

Endpoint	Control	Cu-only	Ni-only	Cu-Ni
Female HSI	5.01 ± 0.39^{a}	4.42 ± 0.59^{a}	4.53 ± 0.64^{a}	4.18 ± 0.41^{a}
Female GSI	12.26 ± 2.11^{a}	15.81 ± 2.39^{a}	11.84 ± 1.27^{a}	16.11 ± 2.32^{a}
Cumulative Total Eggs	3144^{a}	775^{bc}	1348^{b}	434^{c}
Mean Eggs per trio	90.5 ± 9.6^{a}	40.7 ± 16.8^{b}	47.1 ± 15.1^{b}	30.1 ± 17.3^{b}
Spawning events per trio	5.9 ± 0.4^{a}	3.6 ± 1.5^{b}	5.6 ± 0.9^{a}	3.0 ± 0.6^{b}
Time to hatch (days)	5.0 ± 0.3^{a}	5.1 ± 0.4^{a}	5.0 ± 0.7^{a}	4.0 ± 0.6^{a}
Egg Size (mm)	1.31 ± 0.09^{a}	1.28 ± 0.01^{a}	1.36 ± 0.01^{a}	1.35 ± 0.01^{a}
Fertilization Success (%)	97.5 ± 1.5^{a}	94.9 ± 4.8^{a}	96.9 ± 0.5^{a}	97.3 ± 0.8^{a}

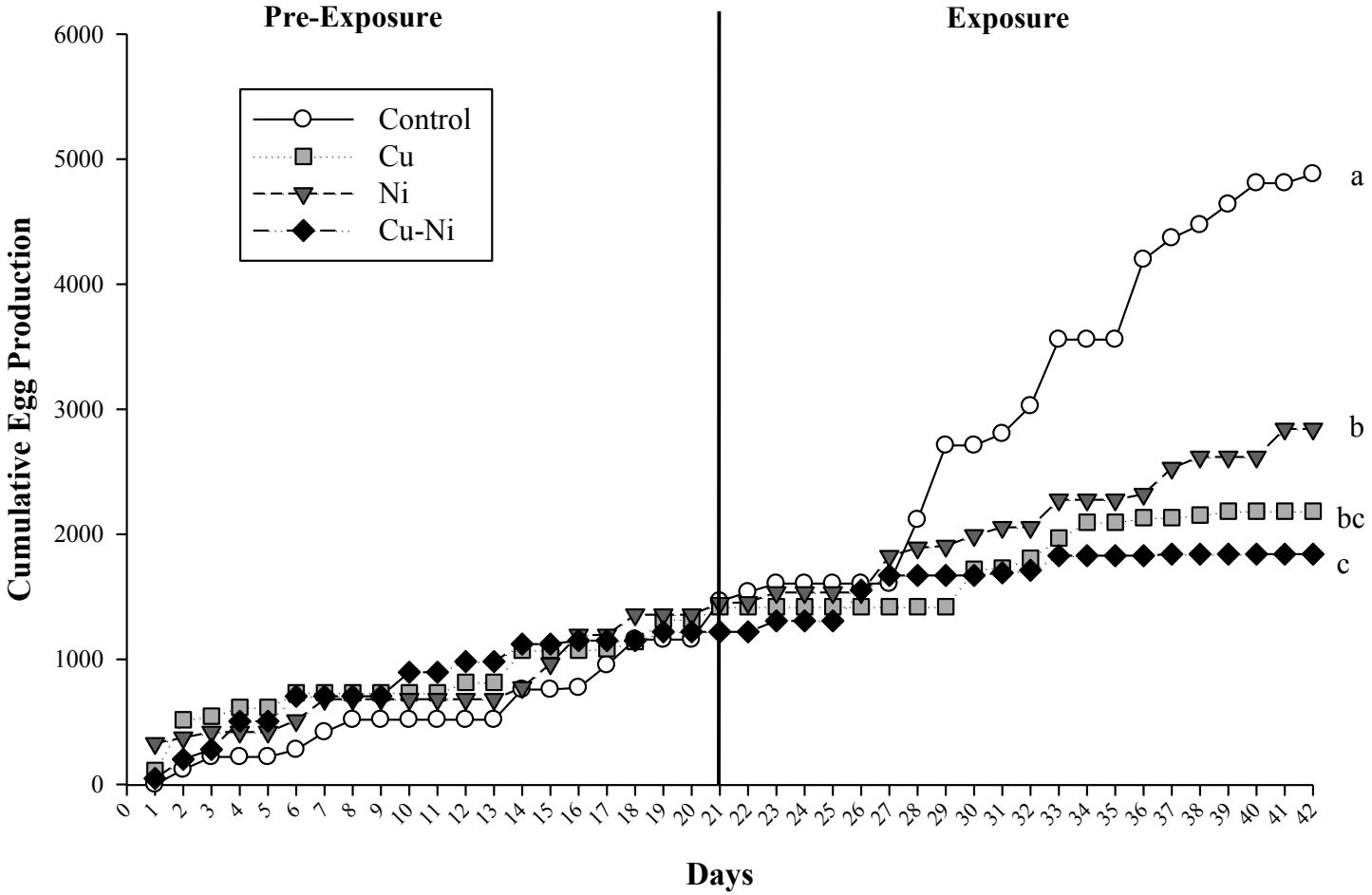

Figure 5.1: Cumulative egg production in fathead minnow breeding trios over 21 days of pre-exposure, and 21-days of exposure to waterborne Cu and Ni, individually and in mixture. Data were analyzed using Kolmogorov–Smirnov (K-S) test. Different letters denote significant differences among treatments, where $p<0.008$ (Bonferroni Correction; n = 5-6 trios) are considered significant.

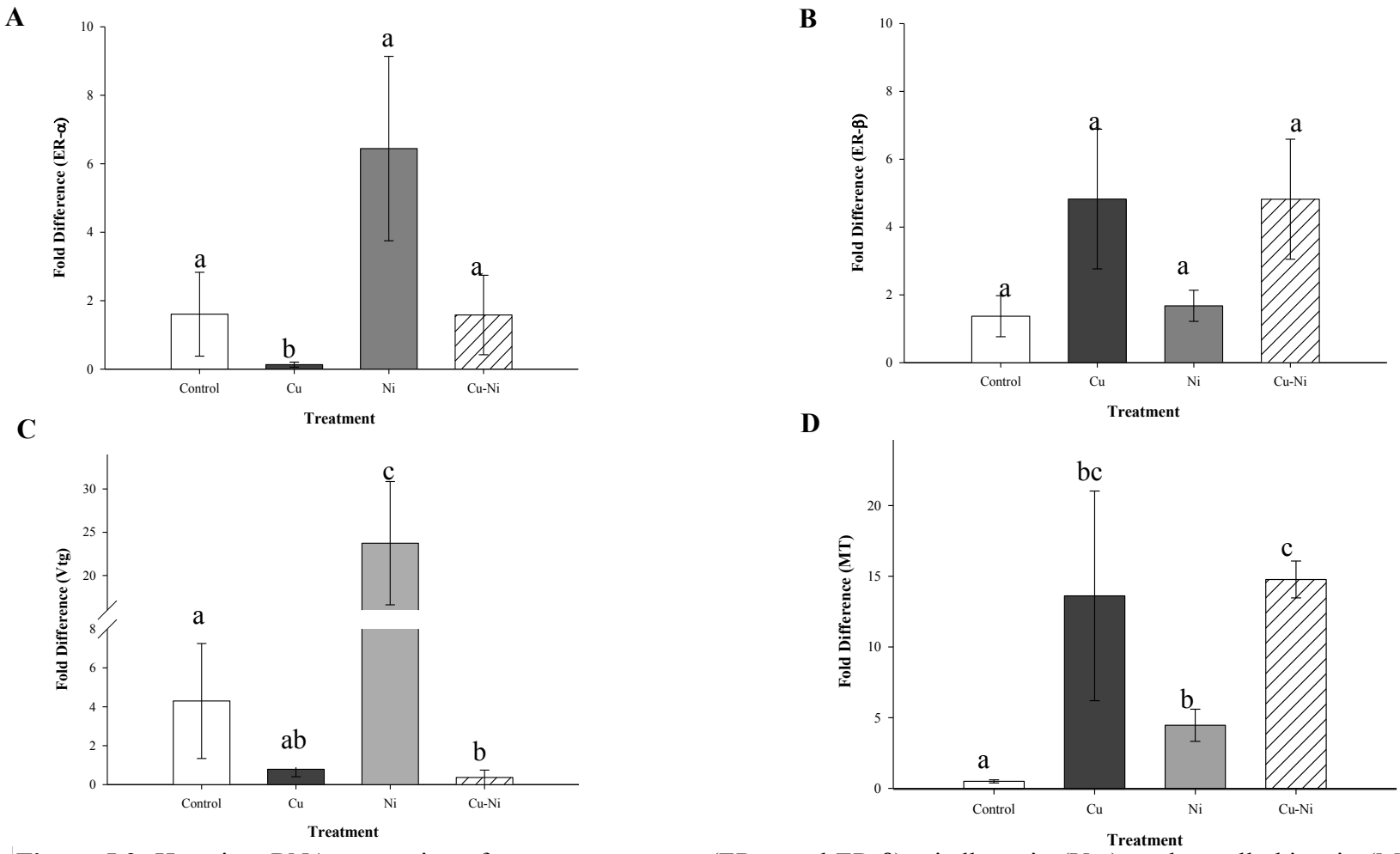

Figure 5.2: Hepatic mRNA expression of estrogen receptors (ER-α and ER-β), vitellogenin (Vtg), and metallothionein (MT) in female fathead minnow following 21 days of exposure to waterborne Cu and Ni, individually and in mixture. Transcript abundance of each target gene is expressed as relative to the housekeeping gene, β-actin. Data are presented as mean ± SEM (n = 5). ER- β, Vtg and MT expression data were analyzed for statistical significance using 2-way ANOVA, and ER- α data were analyzed by Scheirer-Ray-Hare test. Significant differences ($p \leq 0.05$) among treatments are indicated by different letters.

5.3.4. Plasma estradiol levels and ovarian histology

Circulating female estradiol concentrations was not affected by exposure to Cu, alone or in mixture with Ni (Fig. 5.3). However, a significant decrease (~40%) of plasma estradiol level was observed in fish exposed to Ni alone (Fig. 5.3).

The standardized histopathological examination of ovarian sections indicated a general trend in the abundance of different follicular stages (post-vitellogenic > pre-vitellogenic > atretic) across treatments except in the metal mixture treatment, where a different pattern (pre-vitellogenic > post-vitellogenic = atretic) was observed (Fig. 5.4). No significant differences were recorded in the proportion of each follicular stage across different experimental treatments, with the exception of a significant decrease in post-vitellogenic follicles and an increase in the atretic follicles in the metal mixture treatment relative to the control (Fig. 5.4E).

5.3.5. Tissue-specific metal accumulation

We examined the accumulation of Cu and Ni in the gill, liver, gonad, and carcass of female FHMs. Exposure to Cu, alone or in combination with Ni, significantly increased Cu accumulation in all tissues except in the ovary relative to the control (Figure 5.5). There was no significant difference in Cu levels in any tissues between the Cu only, and Cu and Ni mixture treatments, except in the carcass where the Cu level was significantly higher in the mixture treatment relative to the Cu only treatment. The increase in Cu accumulation was highest in the liver (5-6 fold), followed by the gill (~3 fold) and carcass (~2 fold). The tissue-specific Cu levels in fish exposed to Ni alone were found to be similar to that in the control fish.

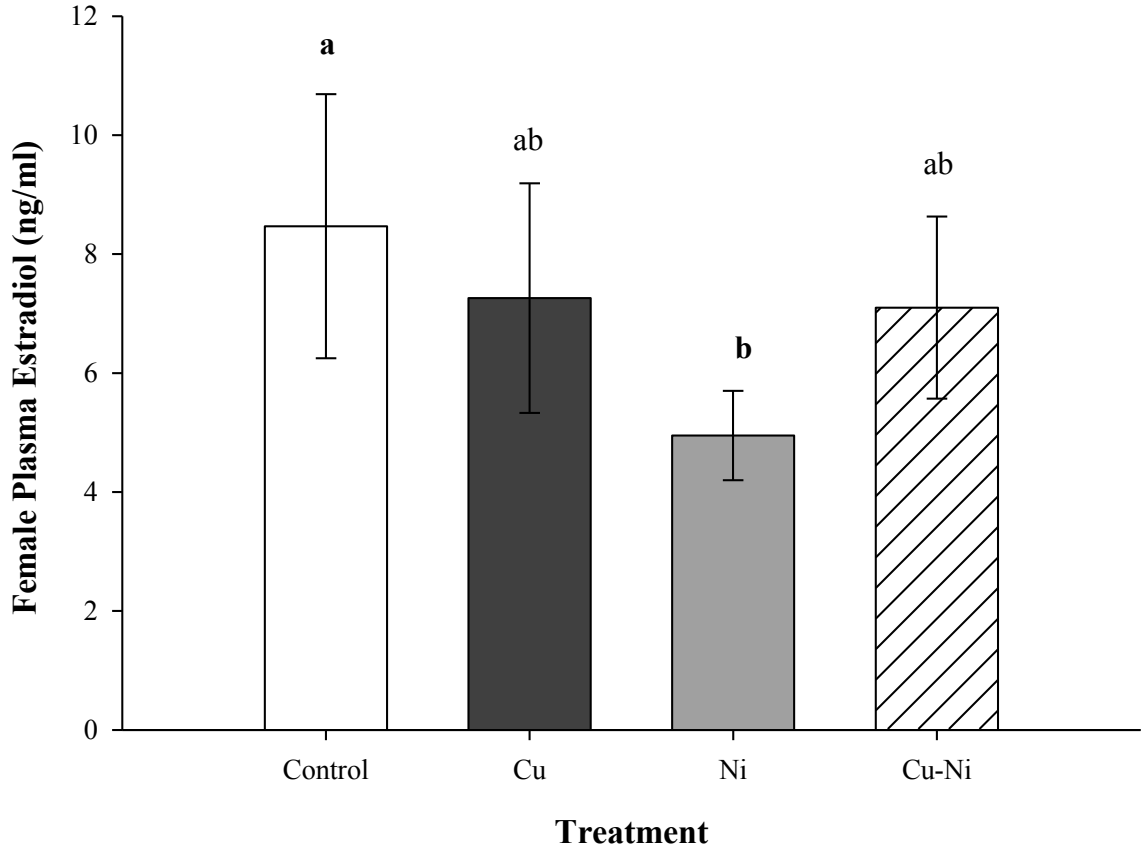

Figure 5.3: Plasma estradiol concentration in female fathead minnows following 21 days of exposure to waterborne Cu and Ni, individually and in mixture. Data are shown as mean ± SEM (n = 5). Data were analyzed for statistical significance by 2-way ANOVA, and significant differences ($p \leq 0.05$)

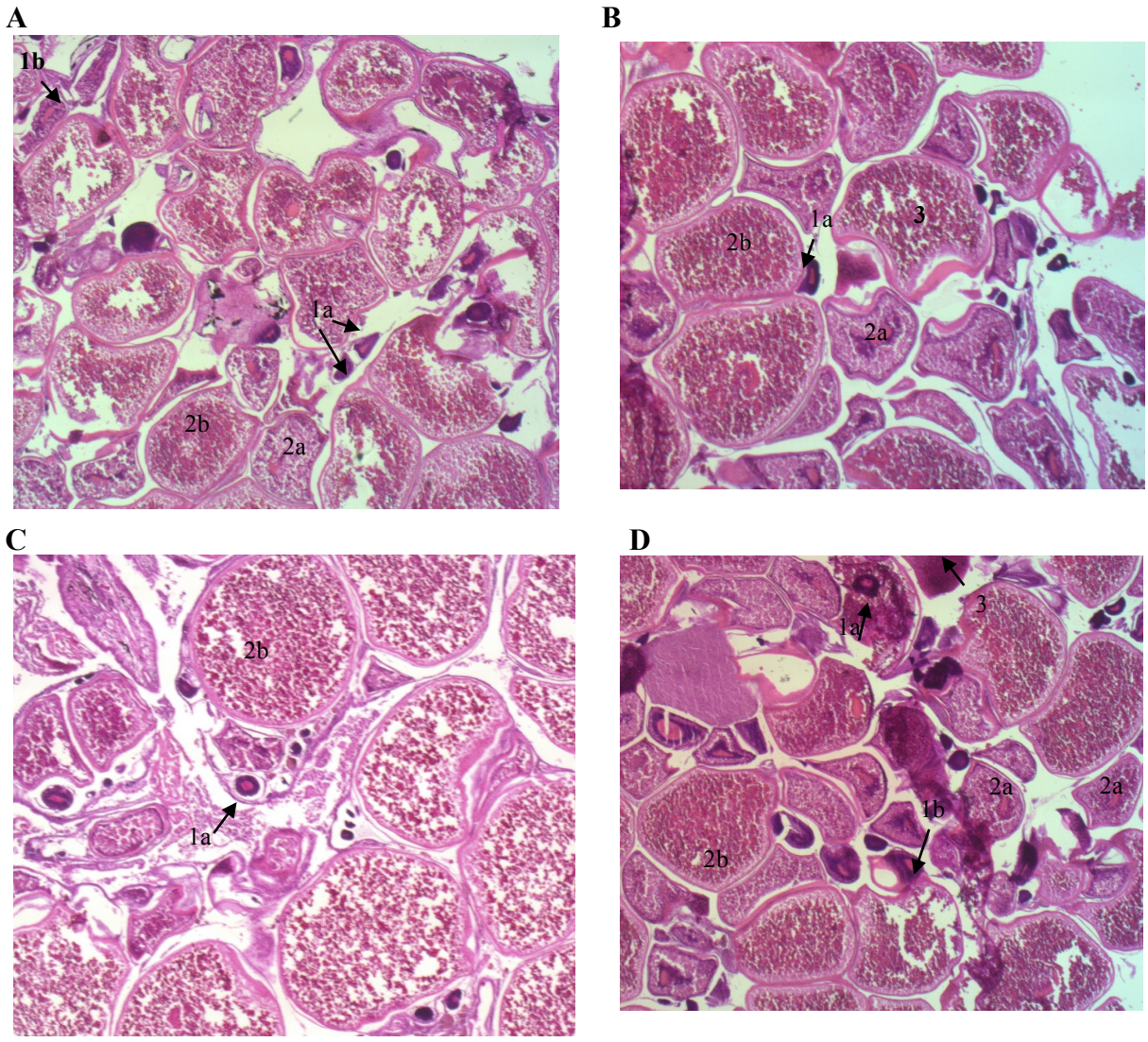

Figure 5.4: Part A. Representative light micrographs (magnification: 2.5X) of the stained sections of ovary in female fathead minnows following 21days of waterborne exposure to Control (A), waterborne Cu (B), waterborne Ni (C), and waterborne Cu and Ni in mixture (D). The follicles in the pre-vitellogenic stage are indicated by 1a (perincucleolar oocyte) and 1b (cortical alveolar), and follicles in the post-vitellogenic stage are indicated by 2a (early vitellogenic) and 2b (late vitellogenic), respectively. Follicular atresia (immature/degenerating follicle) is identified as 3

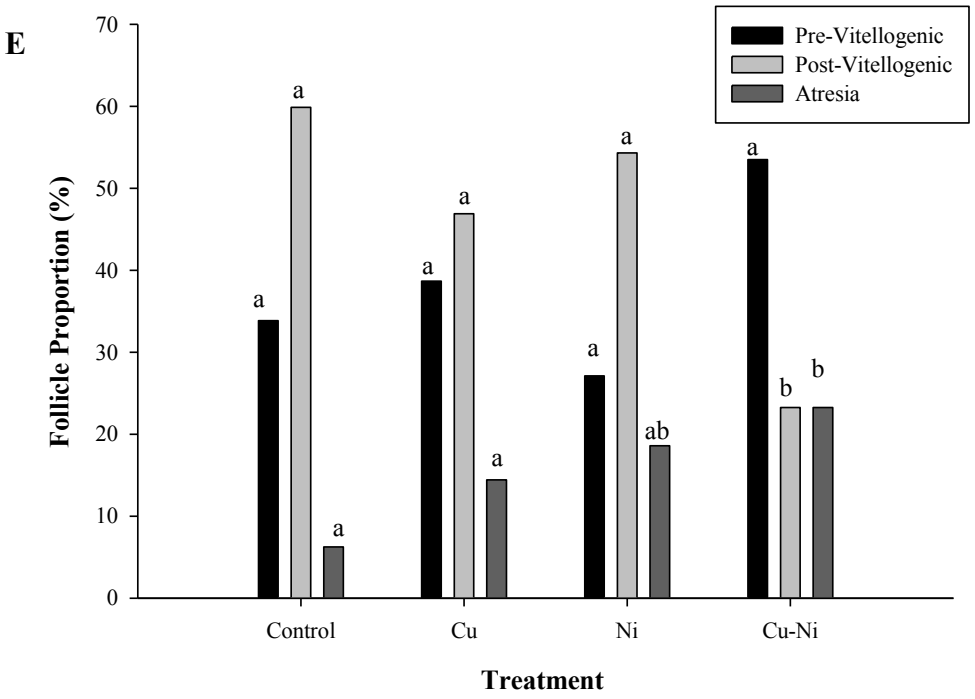

Figure 5.4 Part B (E) Relative proportions of different types of follicles in the ovary. Significant differences ($p \leq 0.05$) among treatments are indicated by different letters (2-way ANOVA).

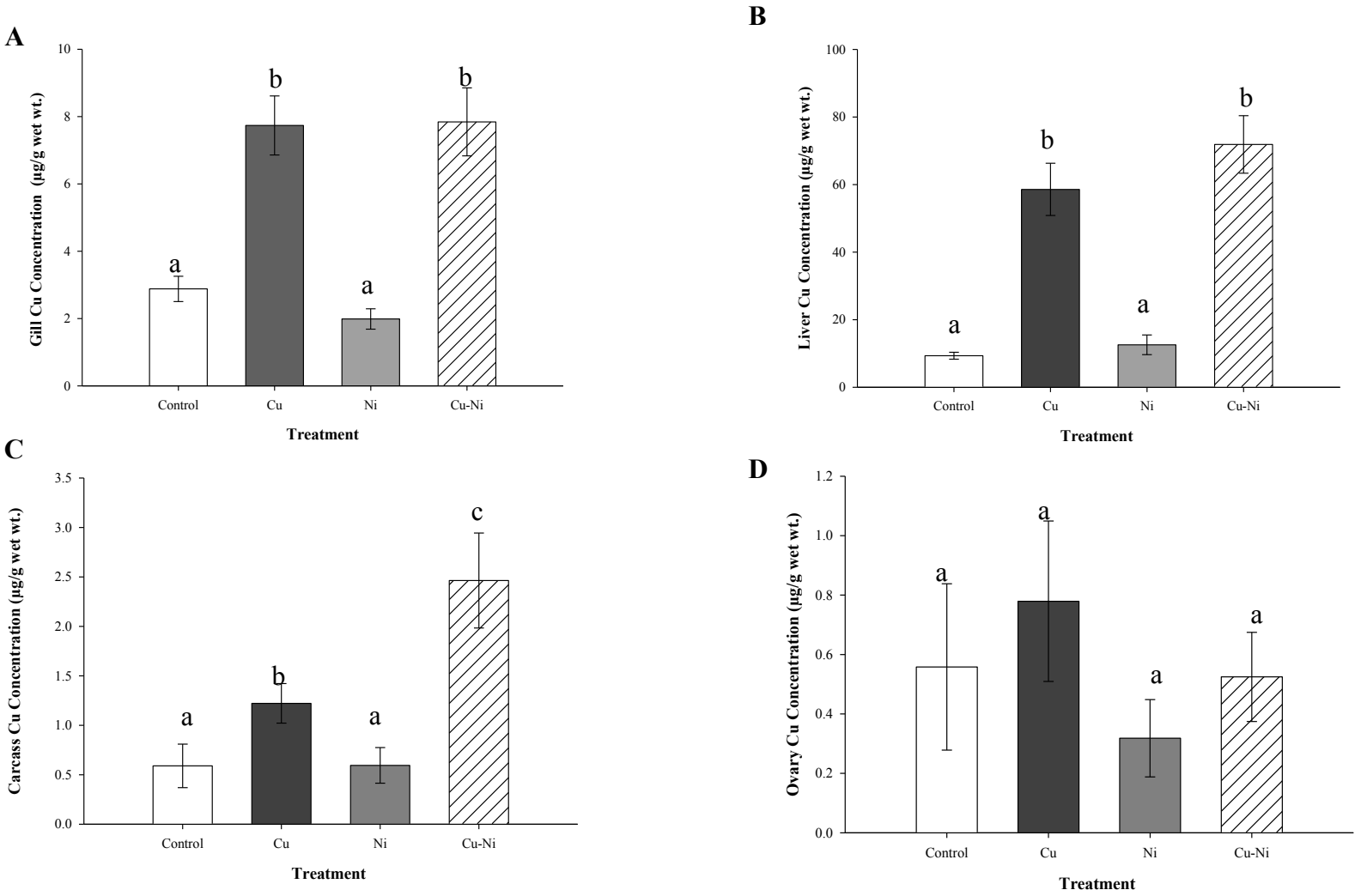

Figure 5.5: Copper concentrations in the gill (A), liver (B), ovary (C), and carcass (D) of female fathead minnows following 21 days of exposure to waterborne Cu and Ni, individually and in mixture. Data are shown as mean ± SEM (n = 8-10 for gill and carcass; n = 5 for liver and ovary). Gill and liver data were analyzed by 2-way ANOVA for statistical significance, and carcass and ovary data were analyzed by Scheirer-Ray-Hare test. Significant differences ($p \leq 0.05$) among treatments are indicated by different letters.

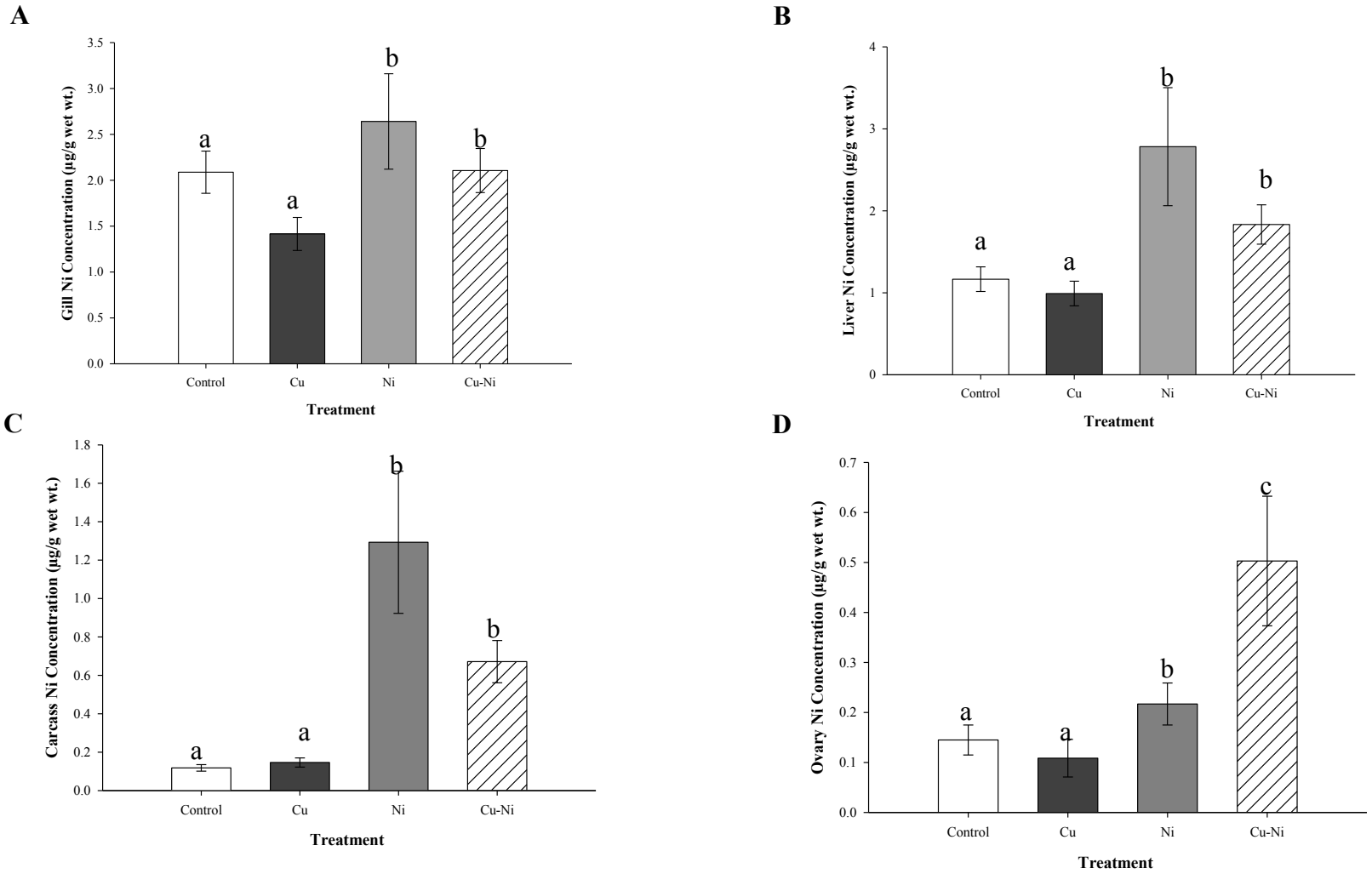

Figure 5.6: Nickel concentrations in the gill (A), liver (B), ovary (C), and carcass (D) of female fathead minnows following 21 days of exposure to waterborne Cu and Ni, individually and in mixture. Data are shown as mean ± SEM (n = 8-10 for gill and carcass; n = 5 for liver and ovary). Gill, liver and ovary data were analyzed by 2-way ANOVA for statistical significance, and carcass data were analyzed by Scheirer-Ray-Hare test. Significant differences ($p \leq 0.05$) among treatments are indicated by

In contrast to tissue-specific Cu accumulation, exposure to Ni, alone or in mixture with Cu, alone or in mixture with Cu, significantly increased Ni burden in all of the tissues examined (Figure 5). The tissue-specific Ni accumulation was similar in both Ni only, and Ni and Cu mixture treatments, except in the ovary where the Ni level was significantly higher in the mixture treatment relative to the Ni only treatment. The increase in Ni accumulation was highest in the carcass (>10 fold), whereas the magnitude of increase was much smaller in all other tissues (2-4 fold). The tissue-specific Ni levels in fish exposed to Cu alone was found to be similar to that in the control fish.

5.4. Discussion

The current study is the first to examine the effects of interactions between chronic waterborne Cu and Ni exposure on tissue-specific metal accumulation and reproductive performance in fish. The concentrations for waterborne Cu and Ni used in the present study are environmentally relevant, as these metals have been found to occur in similar concentrations in highly contaminated natural freshwater systems (ATSDR, 2004; Sancey et al., 2011). The present study demonstrated that the chronic interactions of waterborne Cu and Ni could increase the tissue accumulation of each metal and elicit an additive effect on the reproductive capacity of FHM.

In the current study, the most sensitive reproductive parameter during chronic exposure to waterborne Cu and Ni, alone and in mixture, was fish fecundity (cumulative egg production, and mean eggs/trio). We observed a 75% and 55% reduction in cumulative egg production and mean eggs/trio, respectively, in FHM exposed only to waterborne Cu (45 μg/L) relative to the control fish (Table 5.2). In addition, spawning events/trio was also found to be significantly reduced in FHM exposed to waterborne Cu alone, although fertilization, hatching, and size of the eggs were

not affected (Table 5.2). These observations are consistent with our previous study, where a similar decrease (65-70%) in cumulative egg production and mean eggs/trio were recorded in FHM treated with waterborne Cu (75 μg/L) for 21 days under identical water chemistry (Driessnack et al., 2016). Horning and Neiheisl (1979) also reported a significant decrease of egg production and spawning events in in bluntnose minnows (*Pimephales notatus*) following chronic exposure to waterborne Cu (≥18 μg/L) in moderately hard water (170-230 mg/L as $CaCO_3$).

Similar to Cu, exposure to waterborne Ni alone also produced adverse effects on fish fecundity, although none of the other reproductive endpoints examined including cumulative spawning events was affected (Table 5.2). The cumulative egg production and mean eggs/trio/day decreased by 57% and 48%, respectively, in FHM exposed to waterborne Ni alone (260 μg/L) compared to the control fish (Fig. 5.1; Table 5.2). The chronic reproductive effects of Ni have been investigated sporadically in fish. Pickering (1974) reported that fecundity and egg hatchability were significantly reduced in FHM, but only at concentrations ≥ 730 μg/L. It is to be noted though that that Pickering (1974) used an exposure water with much higher hardness (210 mg/L as $CaCO_3$) compared to that in the present study (155 mg/L as $CaCO_3$), which might have resulted in higher effect concentrations due to reduced bioavailability of Ni (Niyogi and Wood, 2004). Ouellet et al. (2011) also examined the reproductive effects of chronic waterborne Ni (90 μg/L) exposure in FHM in very hard water (520 mg/L as $CaCO_3$), and found no significant effect on fish fecundity although spawning was reduced. More recently, Alsop et al. (2014) reported a significant reduction in egg production in zebrafish (*Danio rerio*) chronically exposed to Ni *via* diet (116 μg/g diet). In general, it appears that the, Ni-induced reproductive effects in FHM observed in the present study are consistent with previous studies.

Co-exposure of Cu and Ni, each at a concentration similar to that in the individual exposures, resulted in a greater reduction in fish fecundity as well as spawning events relative to either of the single metal exposures. The cumulative egg production and mean eggs/trio/day decreased by approximately 86% and 67%, respectively, in FHM exposed to binary mixture of waterborne Ni and Cu relative to the control fish (Fig. 5.1; Table 5.2). In order to evaluate whether the interactions produced additive effects on FHM fecundity, we employed the following predictions of simple additivity, as described in Niyogi et al. (2015) and Norwood et al. (2003):

$$\text{Simple additivity} = [(x + y) - f] \times 100$$

Where x and y are the measured reduction of fish fecundity following exposure to waterborne Cu and Ni, respectively, relative to the control (as % fraction), and f is the interaction factor which is derived by multiplying x and y. Based on this derivation of simple additivity, waterborne Cu and Ni in mixture was predicted to elicit an 89% reduction in FHM fecundity, which was close to the measured response (86% decrease) in the same experimental treatment. This derivation of simple additivity also resulted in a predicted decrease of 76% in mean eggs/trio as opposed to the observed decrease of 67%. In general, it is reasonable to suggest that the interactions of Cu and Ni during chronic waterborne exposure produced a simple additive effect of FHM egg production. However, no similar additive effect of Cu and Ni in mixture was observed with any other reproductive endpoints examined in the present study. Although there is no previous record of additive effects of combined Cu and Ni exposure on fish reproduction, Cu when present in mixture with Cd (like Ni, which also exhibits a different mode of toxic action than Cu in fish (Niyogi and Wood, 2004)) resulted in a greater than additive effect on FHM reproduction (Driessnack et al., 2016).

The interactions of waterborne Cu and Ni may impair reproductive output in FHM by

altering the physiological pathways important for reproduction (a direct effect), and/or by affecting the energy allocation for reproduction (an indirect effect). In the present study, the hepatic expression of three genes (ER-α, ER-β, and Vtg) was evaluated, and each of these genes has important roles in regulating reproductive output in female fish. The development and maturation of eggs in fish requires vitellogenesis, which is initiated by the binding of circulating estradiol to the estrogen receptors located in hepatocytes (Nelson and Habibi, 2010). Binding of estradiol to the estrogen receptor ER-β induces vitellogenin synthesis and subsequently increases the expression of ER-α. This ultimately results in hepatocytes being more sensitive to stimulation by estradiol and ultimately synthesizing more vitellogenin (Nelson and Habibi, 2010). Mammalian studies suggest that both Cu and Ni can act as metalloestrogens and disrupt estrogenic functions by binding to the estrogen receptors (Martin et al., 2003; Dabre, 2006; Aquino et al., 2012). On the other hand, we also examined the hepatic mRNA expression of metallothionein (MT), which is critically involved in the detoxification of metals (Ahearn, 2010). Chronic exposure to metals often leads to the induction of *de novo* MT synthesis (Roesijadi, 1996; Ahearn, 2010). However, it has been suggested that metal-induced upregulation of MT synthesis requires increased cellular energy allocation (Erk et al., 2008), which may also have an indirect adverse impact on fish reproductive output.

In the present study, exposure to Cu alone resulted in a slight downregulation of hepatic ER-α and Vtg genes, but no change in the expression of ER-β gene in female FHM (Fig. 5.1). In our previous study, chronic exposure to waterborne Cu was also found to cause a small decrease in hepatic ER-α expression in female FHM, but no change in Vtg expression was recorded (Driessnack et al., 2016). In addition, in the present study, we also did not observe any change in the circulating estradiol level or ovarian histopathology in female FHM exposed to Cu alone in the

present study (Fig. 5.3 and 5.4). Driessnack et al. (2016) also found no effect of chronic Cu exposure on the estradiol level in female FHM. These findings suggest that the Cu-induced decrease in FHM reproductive output was not likely caused by the disruption of estrogen-mediated reproductive functions. In contrast to Cu, the hepatic expression of both ER-α and ER-β genes seemed to increase following exposure to Ni alone relative to the control (Figure 1), although no statistically significant difference was noted between the two treatments possibly because of the high variability in the data and/or a smaller sample size (n=5) in our analysis. Nonetheless, the apparent increase in the expression of estrogen receptors might have been physiologically relevant, and likely to have led to the stimulation of hepatic Vtg expression. In general, the alterations in ER and Vtg gene expressions probably occurred as a compensatory response in order to sustain reproductive output under reduced stimulation by circulating estradiol following exposure to waterborne Ni (Figure 5.3). It is not clear, based on the observations of our study, how Ni can reduce plasma estradiol level, as exposure to Ni alone did not induce any apparent histopathological damage to the ovary (Figure 5.4). It is probable that Ni might have affected steroidogenesis by interfering with the calcium-mediated signaling pathways (Kasprzak, 1987). However, it is to be noted that the interactions of Cu and Ni seemed to have an antagonistic effect on the circulating female estradiol level, as no difference in estradiol level was observed between the control and the Cu-Ni mixture treatment. Clearly, further research is required to characterize the physiological mechanisms by which Ni may affect estradiol synthesis or metabolism in fish. Nonetheless, it is apparent that the Ni-induced impairment of FHM fecundity was likely caused by the reduction in the circulating estradiol level.

Co-exposure to Cu and Ni did not affect the hepatic gene expression of either of the two estrogen receptors (ER-α or ER-β) as well as the circulating estradiol level in female FHM. These

observations indicate that the reproductive functions in fish that are mediated by estrogen signaling pathways were not influenced by the exposure to Cu and Ni mixture. In contrast, we recorded a marked reduction of the hepatic Vtg gene expression in fish exposed to the binary mixture of Cu and Ni relative to the control, which was associated with a significant increase in hepatic expression of the MT gene (Figure 5.2). Collectively, these findings suggest that the chronic exposure to Cu and Ni in mixture induced the downregulation of vitellogenesis and associated decrease in fish fecundity *via* an indirect mechanism. To this end, it could be speculated that this indirect mechanism might involve the disruption of energy homeostasis, at least in part.

Our study revealed that individual exposures to waterborne Cu and Ni significantly increased the accumulation of respective metals in all tissue-types (gill, liver, ovary, and carcass) examined except the Cu levels in the ovary (Fig 5.5 and 5.6). A similar trend of tissue-specific Cu accumulation has been reported previously in FHM chronically exposed to waterborne Cu (Driessnack et al., 2016). Similarly, elevated Ni levels have been observed in various fish tissues including gill, liver and gonads following chronic exposure to Ni, either *vi*a water or diet (Sreedevi et al., 1992; Canli and Kargin, 1995; Ptashynski and Klaverkamp, 2002). More importantly, we observed that the interactions of waterborne Cu and Ni did not significantly affect their tissue-specific accumulation in FHM, except an increased Cu accumulation in the carcass and Ni accumulation in the ovary (Figure 5.5 and 5.6). The branchial Cu uptake in fish can occur by both sodium-dependent (e.g., epithelial sodium channels) and sodium-independent pathways (e.g., Cu transporter 1 and/or divalent metal transporter 1). In the present study, the sodium-independent pathway is expected to have played a predominant role in branchial Cu uptake, since the sodium-dependent pathway is known to saturate at roughly 6-7 fold lower waterborne Cu level than that used in our exposure (45 μg/L) (Grosell and Wood, 2002; Grosell, 2012). In contrast, the

mechanism of waterborne Ni uptake in aquatic organisms is presently not fully understood, and the circumstantial evidence indicates the potential involvement of magnesium transport pathways (Pane et al., 2004b; Pane et al., 2005). Since the apparent uptake of waterborne Cu and Ni occurs through different cellular pathways, it is not clear how Cu and Ni accumulation in the carcass and ovary, respectively, was stimulated in the mixture treatment. Nonetheless, similar to our observation, Brix et al. (2016) recently reported that Cu uptake in fish gills was stimulated during short-term waterborne exposure to Cu and Ni mixture, although they did not observe any such reciprocal effect on Cu uptake. The stimulation of Ni uptake might have occurred due to the uptake Ni *via* paracellular pathways, as Cu has been found to increase paracellular permeability in both fish and mammalian tissues (Ferruzza et al., 2002; Jonsson et al., 2006).

In the present study, the expression of hepatic MT gene was significantly upregulated in FHM following exposure to Cu or Ni relative to the control. Driessnack et al. (2016) also reported a significant upregulation of hepatic MT gene in FHM during chronic exposure to Cu. Interestingly, however, in the present study, the maximum induction of hepatic MT gene was recorded in fish exposed to the mixture of waterborne Cu and Ni (Figure 5.2). It is apparent that the sharp upregulation of hepatic MT gene was triggered by the combined tissue burden of Cu and Ni in FHM tissues. It is also to be noted that the histopathological damage in the ovary (decreased abundance of post-vitellogenic follicles and increased abundance of atretic follicles) was observed only in fish exposed to the mixture of Cu and Ni (Figure 5.4). This seems to be induced by the histopathological effects of Ni (Athikesavan et al., 2006), since the highest accumulation of Ni in the ovary was observed in fish exposed to the mixture of waterborne Cu and Ni relative to that in all other treatments (Figure 5.6).

5.5. Conclusion

The present study demonstrated that chronic waterborne exposure to Cu and Ni in binary mixture elicits an additive effect on the reproductive output in FHM. The additive effect of Cu and Ni on fish fecundity was found to be essentially induced by the downregulation of vitellogenesis and histopathological damage to the ovary. The interactions of Cu and Ni were also found to increase the tissue-specific accumulation of both metals during chronic waterborne exposure. The concurrent increase in tissue burden of Cu and Ni corresponded with the maximum observed upregulation of hepatic MT gene, indicating an increased metabolic cost of coping with elevated Cu and Ni body burden. Overall, these findings suggest that waterborne Cu and Ni in mixture may cause additive effects on reproductive capacity in FHM by inducing histopathological damage in ovarian tissue, and potentially also by disrupting energy homeostasis.

CHAPTER 6:

GENERAL DISCUSSION

6.1 Project rationale and goals

As societies continue to develop and expand, so do our demands and impacts on natural ecosystems. While there are no doubts industries, such as base metal mining, are of economic importance, the potential for anthropogenic activities contributing to environmental damage are real and relevant. Knowing the potential for industries to release persistent complex mixtures into ecosystems, effluent discharge is regulated and held to standards of release to protect organisms and receiving environments. In many cases, the requirements that effluents need to meet to be approved for discharge are based on simplified, acute and single contaminant toxicological studies. There is doubt though in the applicability of acute single metal laboratory exposure data to effluents that are chronic, low-dose mixtures in real world receiving environments. The potential for mixtures to have long-term interactive effects on organisms is not a new concept. However, it is one often overlooked. Therefore, the overall objective of this research was to begin to address how mixtures may interact and influence ecologically important endpoints, specifically fish reproduction.

The experiments conducted in this thesis sought to assess the effects of waterborne binary metal mixtures on the reproductive output of a well-established laboratory model fish species, the FHM, across multiple physiological levels. This allowed an in-depth assessment of metal interactions across a range of interconnected endpoints (*e.g.*, egg production, gonad histology, expression of genes that regulate fish reproduction), while simultaneously expanding chronic single metal toxicity knowledge. A secondary objective was to assess the interactive effects of metal mixture exposure on tissue-specific metal accumulation patterns and how tissue metal

burdens might influence fish reproductive capacity. Lastly, considering the results of the FHM reproductive assays, studies were conducted to determine if metals could alter steroidogenesis in isolated ovarian tissues, and if varying the metal mixture ratios modulated any observed responses. It is also pertinent to mention that during the initial literature reviews for experimental design and selection of metals for the mixture studies, it became evident that the reproductive effects of the individual metals, particularly Zn and Ni, are in many ways still unclear, allowing this thesis to fill data gaps in our understanding of reproductive toxicity of both chronic single metals and binary metal mixtures. Overall, this research was aimed at demonstrating the need for metal mixture toxicity assessment, and to provide guidance in how the inclusion of reproductive endpoints may highlight previously overlooked factors of consideration for long-term fish population stability.

6.2 Project summary

6.2.1 Single metal reproductive effects

6.2.1.1 Reproductive effects of cadmium

It was initially difficult to determine if Cd was influencing the number of viable eggs produced by fish, despite molecular and biochemical alterations being observed. One of the factors, that was often considered during experimental design, was the inherent variability in fish reproductive output. This variability may have masked reproductive effects, especially in the case of Cd. Variability in unexposed fish is not uncommon but it can be a complication, and subtle effects can only be discerned by a large sample size or multiple trials. As such the production of all the FHM trios was assessed by averaging the respective treatments together for control and Cd, as if they had been run simultaneously, and statistical analysis indicated that Cd indeed significantly impaired reproductive output in FHM ((Fig. 6.1; K-S Test, $p = 0.017$). The result of the analysis of the combined treatments suggests Cd-induced reproductive toxicity at low doses, and that these effects may be subtle in nature and require consideration of a combination of

endpoints beyond just egg production to draw appropriate conclusions. Somewhat similar to this observation, Wang et al. (2014) also found that the effect of Cd on FHM reproductive output was not evident until pre-exposure baseline egg production was combined with the egg production during exposure in the statistical analysis.

6.2.1.2 Reproductive effects of copper

The two Cu-only *in vivo* studies were run using two different, but environmentally relevant, exposure concentrations. In the first study (Chapter 2), Cu at ~75 µg/L was found to induce physiological changes contributing to changes in energy allocation patterns that potentially affected reproductive performance. In the second study (Chapter 5), Cu at ~45 µg/L also seemed to cause changes in energy allocation, but, there was also strong evidence that Cu alone could alter steroid hormone signaling pathways.

It has been suggested previously that a Cu-induced reproductive effects in fish could occur due to its direct physiological effects (e.g., oxidative damage), and not by direct interactions of Cu on reproductive pathways (Hogstrand 2012). Interestingly, the findings from my *in vitro* study also indicated that Cu impairs the production of estradiol in ovarian explants. Collectively, my *in vivo* and *in vitro* observations imply that the mechanisms of Cu-induced reproductive impairment may shift with altering Cu exposure levels. Copper-induced reproductive toxicity at low exposure levels is likely caused by the interference of Cu with different sites crucial to reproductive signaling, but as the exposure levels increase, so does the degree of oxidative damage, which requires more energetically expensive detoxification, ultimately leading to the reduction of energy allocation for reproduction.

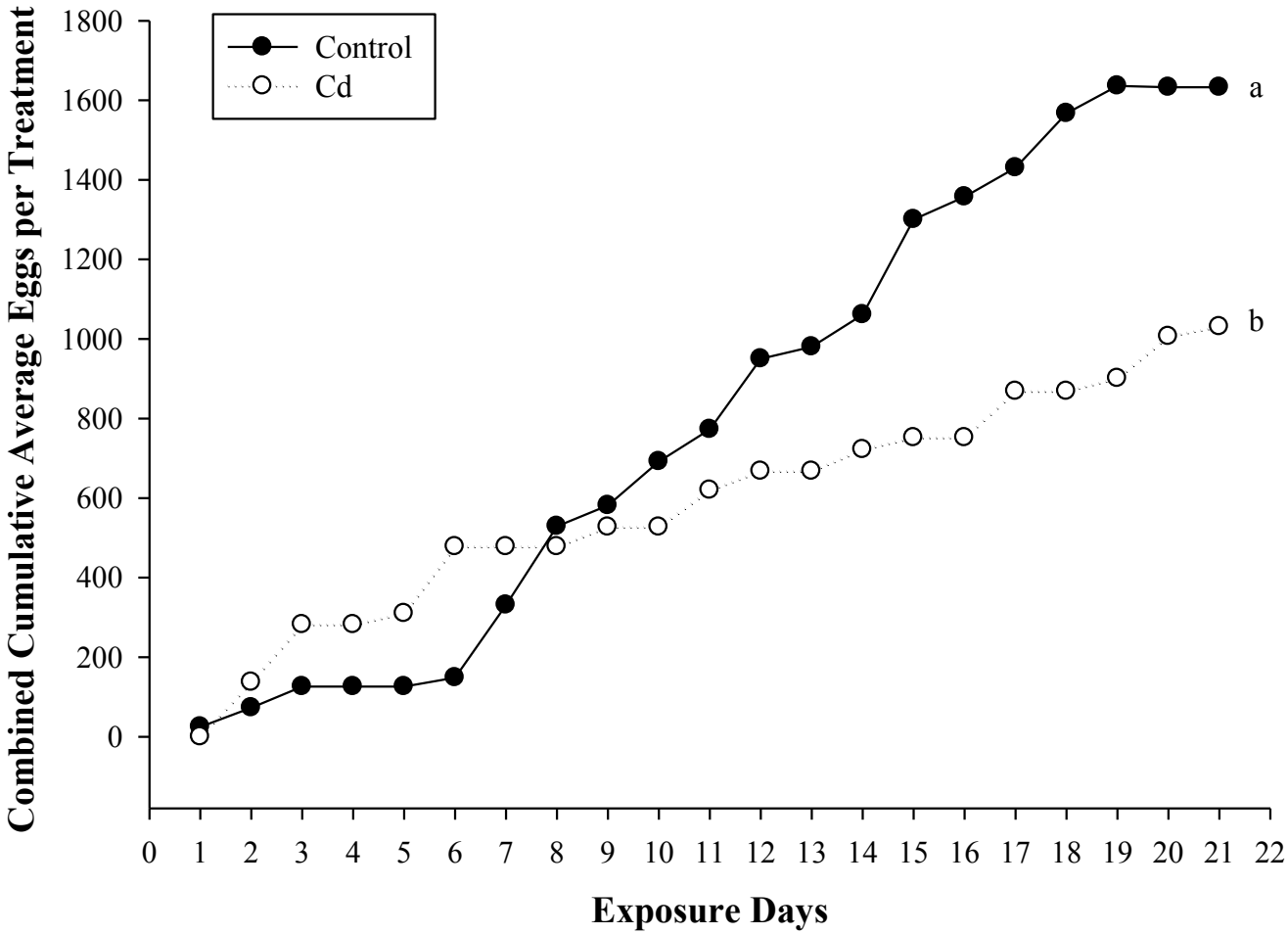

Figure 6. 1 Multiple study combined average cumulative egg production in fathead minnow breeding trios over 21 days of pre-exposure, and 21-days of exposure to control water or waterborne Cd-only. Data were analyzed using Kolmogorov–Smirnov (K-S) test. Different letters denote significant differences among treatments, where $p<0.008$ (Bonferroni Correction; n = 10-14 trios) are considered significant.

6.2.1.3 Reproductive effects of zinc

The assessment of the reproductive effects of Zn in this thesis provided some novel findings. To date, very little is known about how Zn can affect reproductive endpoints including fish fecundity. The results of the *in vivo* (Chapter 3) and *in vitro* (Chapter 4) studies with Zn highlight, for the first time, the ability of Zn to impair not only egg production, but also estradiol production and hepatic Vtg expression. To my knowledge, these observations reveal the potential mechanisms by which Zn at environmentally relevant concentrations can impair fish reproduction. Another interesting observation with regards to Zn exposure was the extreme fragility of the spawned eggs, presumed to be due to a lack of proper water hardening. This effect is one that could be of concern in natural environments, where any external contact with the eggs could result in their rupture and loss. This may occur especially when male FHMs use their fatpads to clear debris and contamination from the spawned eggs. The factors responsible for this egg fragility are worthy of further examination, but may involve the lack of vitelline envelope activation and subsequent conversion to the chorion (Kusa 1957, Arnoult et al. 1996, Ha and Iuchi 1998). Additionally, the vitelline envelope is comprised of zona pellucida (ZP) proteins, and their production in the liver and ovary is ER-dependent and regulated by estradiol (Wu and Zhou 2012). Thus, the egg fragility could be an indirect effect of Zn-induced reduction of estradiol production in fish.

6.2.1.4 Reproductive effects of nickel

Similar to Zn, very little was known about the reproductive effects of Ni in fish prior to this study. In general, the results of my study indicate that Ni can reduce reproductive output in FHMs at an environmentally relevant concentration (Chapter 5). My study also demonstrated for the first time that Ni can reduce the circulating estradiol level and can also alter the hepatic Vtg

expression in fish. Since the mechanistic knowledge of Ni toxicity are in general poorly documented, it is challenging to deduce how Ni-induced reproductive change might have occurred in FHM. Such changes are likely driven mainly by the reduction in serum estradiol, and thereby disrupting the estrogen-mediated reproductive pathways in fish. Nonetheless, the mechanisms by which Ni causes reproductive toxicity in fish remains unclear, and further studies are needed in order to gain a more in-depth knowledge of this aspect.

6.3 Interactive effects of metals in binary mixture

6.3.1 Reproduction

The main objectives of my current research were to determine which combination(s) of metals would elicit the maximum toxicological effects. One of the major strengths of this thesis is that all of the selected metal mixture exposures were conducted using exposure levels of each metal that are environmentally relevant, and thus the findings of this study have real world significance. By comparing the key findings of the three FHM *in vivo* reproductive bioassays conducted in this thesis, a generalized effect scenario can be assembled. Chronic waterborne binary metal exposures can significantly impair the reproductive capacity of FHM in a manner that is additive (Cd-Zn, and Cu-Ni) or greater than additive (Cd-Cu). It should also be noted here that the studies demonstrated that metals in mixture (e.g., Cd and Zn) could produce reproductive toxicity in fish, to a degree greater than any single metal was found to cause reproductive toxicity during individual exposure. From the regulatory perspective, this has important implications since such effects should be accounted for with any future regulatory assessment of metal-mixture toxicity in aquatic ecosystems. Overall, fish fecundity (cumulative egg production) was consistently found to be the most sensitive endpoint assessed in all of these studies, which agrees with the conclusion

drawn by Suter et al. (1987) that fish fecundity is one of the most sensitive endpoints for chronic toxicity assessment in fish.

Identification of sensitive reproductive endpoints such as the cumulative egg production was only one of the major components of this research. This assessment of underlying driving factors of the reproductive toxicity sought to elucidate the factors leading to changes in cumulative egg production. The impaired reproductive output observed in my study following treatment with metal mixtures is postulated to be the result of two primary factors. First, the increased metal exposure was found to alter reproduction by influencing the molecular and physiological parameters linked with fish reproductive functions. One of the notable changes recorded most commonly in relation to this was decreased serum estradiol induced by metal mixtures, an observation that was also supported by the findings from my *in vitro* steroidogenesis assays in isolated ovary explants. In addition, associated effects such as increased follicular atresia, and altered ER expressions and reduced Vtg gene expression in the liver also indicated disruption of estrogen-mediated reproductive functions in FHM. The second contributing factor appeared to be the diversion of energy away from reproduction to detoxification mechanisms, as interpreted from the observed increased tissue-specific metal burden and hepatic MT expression. Overall, these findings suggested that in addition to fish fecundity, sub-organismal endpoints, such as plasma estradiol, hepatic Vtg, and MT expressions, and ovarian histopathology, could also be useful biomarkers for assessing the reproductive toxicity of metal mixtures in fish.

In a general sense, each combination of metals was chosen in this study based on their apparently similar (e.g., Ca –antagonists, Cd, and Zn) or different modes of toxic action (e.g., Ca and Na antagonists, Cd, and Cu), as characterized under acute exposure scenarios. I hypothesized that metals with different modes of toxic actions (e.g., Cd and Cu, or Cu and Ni) would produce

more than additive toxic effects, whereas metals with similar mode of toxic action (e.g., Cd and Zn) would elicit less than additive or additive toxic effects. The results of this thesis indicate that when considering chronic endpoints such as reproduction, metals in binary mixture tend to elicit additive toxicity (e.g., Cd and Zn, Cu and Ni) irrespective of their apparent modes of toxic action. This might have occurred because metal mixtures examined in this study were generally found to induce reproductive toxicity primarily by the combination of altered homeostasis, and reduced circulating estradiol levels and/or downregulation of hepatic vitellogenesis.

6.3.2 Tissue-specific metal accumulation

The research conducted in this thesis provided insights into the interactive effects of metals in mixtures on tissue-specific metal accumulation in fish during chronic waterborne exposure. The assessment of tissue-specific (gonad, gill, liver and residual carcass) accumulation of the four metals in FHM indicated that the interactive effects of metals are more likely to be observed in the liver than in any of the other tissues examined. This is not unexpected since liver is the target organ for many metals and has an important role in maintaining homeostasis through uptake, metabolism, storage, and redistribution of nutrients and other endogenous molecules (Hinton et al. 2001, Hinton et al. 2008).

An antagonistic interaction was observed with Cd and Cu, which are not known to share common uptake pathways, with significantly reduced accumulation of Cu in the liver in the presence of Cd. On the other hand, Cd and Zn are known to have common uptake mechanisms, and Zn was noted to reduce Cd accumulation in the liver, in addition to the gill, during co-exposure. Nonetheless, it is important to note that though these antagonistic interactions on tissue burden did not translate into an ameliorative response on reproductive output in fish. In contrast, during co-

exposure to Cu and Ni, which are known to be absorbed via different pathways in fish, an interactive effect on Ni burden in the ovary was observed as the Ni burden doubled compared to that in the Ni-only treatment. No significant interactive effect of Cu and Ni was observed in any other fish tissues examined, although a consistent trend of Cu and Ni altering the other metal's concentration was noted. Overall, these findings indicate the interactive effects of metal mixtures on tissue metal accumulation during chronic exposure is complex, and cannot always be predicted simply on the basis of our current understanding of their branchial uptake mechanisms in fish. Suggesting that the combined tissue burden of both metals during co-exposure, irrespective of specific metal mixture combinations, played a significant role in mediating reproductive impairment in fish, essentially by increasing the detoxification cost (as reflected by the upregulation of hepatic MT expression) and thereby potentially altering the energy allocation for reproduction.

6.3.3 Environmental context

One question that arises from this research is that what are the general regulatory implications of these findings? In the case of this thesis, the consideration of mechanistic metal interactions were considered, and the results showed that metals, when combined, elicited a greater detrimental effect on reproduction than single metal exposures. Current water quality guidelines, which are usually based on single metal acute toxicity data, likely do not protect exposed systems to the degree assumed. This means that if guidelines are set to protect 95% of species, and only single metal databases are used, we may in fact only be adequately protecting 50-75% of the species, using hypothetical percentages as an example.

Natural systems are not like a laboratory and despite best efforts to extrapolate data or use site-specific guidelines, until we can understand how and where metal interactions occur in fish, the current guidelines will not be very effective in the natural systems. One of the emerging approaches has been the use of Adverse Outcome Pathways (AOPs) to try and use the available mechanistic data to link molecular/cellular changes (initiating events) to higher level endpoints such as reproduction. This is essentially a bottom-up approach reframed using the most recent advances in high-throughput, high-output techniques. However the results are a single snapshot of one moment in an artificial scenario. A return to or greater attention paid to top-down approaches, as used in ecology, still very much have a place in toxicology (Beketov and Liess 2012).

The expression of reproductive genes and circulating sex steroid hormone levels are sources of invaluable data, however, in our attempts to understand all the inner workings of fish physiology, we should not forget the bigger picture. That may mean including more histological assessment of gonadal tissue to see how the oocytes are affected and in turn how the health of offspring is impacted. The results of this thesis have highlighted a number of potential mechanistic interaction sites for metals in the fish reproductive pathway, but it also highlights that cumulative egg production is the most sensitive endpoint assessed during metal-mixture exposure. In many ways, this thesis did follow the trends of current AOP models. However, the focus in the *in vivo* studies was always centered around the quantity and quality of the eggs produced by fish. This thesis was not developed to solely identify where metals may interact within the organisms; it sought to also consider reproduction as a whole from a chronic perspective, with an understanding of long-term population stability as the goal. The following section highlights future research considerations to help further this avenue of research.

6.4 Future considerations

The research detailed here provides valuable insights into the effects of single metal exposures as well as their interactive effects in binary metal mixtures on fish reproductive performance and tissue-specific metal accumulation. However, my research also brought up several aspects for future research considerations, in order to enhance our understanding of metal mixture toxicity in fish. These aspects are outlined below:

6.4.1 Expansion of the fathead minnow reproductive bioassay

The FHM reproductive bioassay employed in my study can be adopted to assess the toxicity of tertiary or even quaternary mixtures of metals. These bioassays could also be employed to assess the contribution of dietary metal mixture exposure on fish reproductive performance using spiked commercial diets or more ideally using cultured invertebrate species (*e.g.*, *Chironomids*) raised under metal-mixture exposure. The ideal assessment scenario would be a simultaneous waterborne and dietary exposure to metal mixtures, truly mimicking a natural exposed system. Moreover, as it has been mentioned extensively throughout this thesis, it is important to assess the chronic metal-mixture toxicity under variable dose ratios of metals in the exposure, although this would be a tedious and expensive research endeavor. Finally, future investigations of metal-mixture toxicity using the FHM bioassay may consider employing a more extended exposure period (longer than 21 days), which may help to differentiate the more robust effects from the transient ones, as observed in this study (*e.g.*, spawning frequency, fertilization success).

6.4.2 Expansion of *in vivo* endpoints

Despite a wide range of endpoints across a range of biological levels assessed in my study, there were endpoints excluded from this thesis that could have provided further mechanistic information for the observed effects. In particular, future research in this area should focus on assessing the oxidative damage endpoints, which would provide valuable support for the potential linkage between altered energy allocation and reproduction impairment. Increased oxidative damage coupled with increased MT protein content would both be valuable lines of evidence supporting the hypothesis that changes in energy allocation due to metal detoxification, and toxicity is a major contributing factor for impaired reproductive performance. In addition, future studies may benefit from ovarian histopathological assessment by immunohistochemical (IHC) techniques. The advantages gained by using IHC would include the localization and estimation of Vtg quantity within the oocytes, which might help to further explain the effects of metal-mixtures on fish fecundity.

Other contributing factors to reproductive impairment observed in my study may include the effects of metal-mixtures on the male fish, leading to reduced fertilization success and/or egg hardness, which may ultimately result in decreased egg production. Male endpoints could include quantification and measurement of nuptial tubercules, plasma KT-11 concentrations, testes histopathology. In addition, future investigations should also focus on assessing the effects of metal mixtures on male reproductive behaviour (e.g., courtship, nest care), since such effects could also contribute to decreased reproductive performance.

Future investigations should also include changes in expression of genes related to HPG (hypothalamus-pituitary-gonad) axis, particularly in the brain. The current knowledge on the

accumulation and effects of metals in the fish brain is limited. However it is a key component in the complex signaling pathways (mediated by LH, FSH, and GnRH) that regulate reproduction. In addition, examination of potential changes in the expression of StAR and P450arom proteins in the ovary would also be helpful in further delineating the pathways involved in metal-mixture induced reproductive toxicity in fish. With regards to tissue-specific metal accumulation, future work should include the kidney, since the kidney serves as the long-term storage site for metals, especially for Cd and Ni, during chronic exposure.

6.4.3 Expansion of *in vitro* assay techniques and endpoints

The potential research avenues that could be pursued in the *in vivo* assays are extensive and ultimately costly endeavors, an issue that can be addressed using more cost effective *in vitro* approaches. The ovarian explant assay was an asset to the thesis, and much like the *in vivo* reproductive tests, this could be expanded in a range of directions. For example, this could include evaluation of the steroidogenic effects of metal-mixtures in testicular tissue. Future *in vitro* investigations should also include evaluation of the expression of key genes involved with steroidogenesis in both ovary and testis of fish.

Another type of *in vitro* assessment to be considered would be the use of multiple-tissue co-cultures, as detailed by Johnston et al. (2014), which would allow for a more in-depth assessment of metal-mediated impacts on the HPG axis. This may help in identifying the key steps in the HPG pathway that lead to the attenuation of steroidogenesis.

6.5 Conclusion

The results of the research detailed here clearly demonstrate that binary metal mixtures can alter reproduction output in FHM, with strong evidence of interactive effects (additive and greater

than additive). The *in vivo* data provide strong evidence that cumulative egg production in FHM is a significant and consistent chronic endpoint for assessing the reproductive effects of waterborne binary metal mixture exposures. This thesis also indicates that although the underlying factors contributing to diminished egg production may include multiple endpoints (hepatic ER and VTG expressions, serum estradiol, increased follicular atresia), the overall metal-mixtures exposures elicit their reproductive toxicity predominantly by their indirect effect on estrogen-mediated reproductive pathways in fish. This indirect effect appears to be induced by elevated metal burden in target tissues - increasing the energetic cost of metal detoxification, and suppressing estradiol synthesis and/or vitellogenesis. The findings of my thesis also suggest that the interactive effects of metal mixtures during chronic waterborne exposure on the accumulation of metals in fish are complex, as the patterns of accumulation seem to depend on the combinations of metals in the mixture and also the tissue type.

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APPENDIX[a]

[a] Supplementary information for this thesis are provided in this Appendix. Data include a summary of the water quality parameters assessed during each of the *in* vivo reproductive bioassays (Chapter 2, 3, and 5).

Table A1: Summarized general water quality parameters measured during the 21-day fathead minnow reproductive bioassays. All values are listed as mean ± SE, no statistical differences were noted for any of the treatments.

	Temperature (°C)	pH	Hardness (mg/L)	Ammonia
Cd-Cu Study				
Control	23.5 ± 0.1	7.5 ± 0.05	166.4 ± 1.6	0.01 ± 0.01
Cu-only	24.0 ± 0.1	7.3 ± 0.05	165.0 ± 1.6	0.01 ± 0.01
Cd-only	24.1 ± 0.1	7.3 ± 0.04	169.4 ± 2.2	0.01 ± 0.01
Cd-Cu	23.9 ± 0.1	7.4 ± 0.04	168.0 ± 2.0	0.01 ± 0.01
Cd-Zn Study				
Control	25.3 ± 0.1	7.4 ± 0.02	147.1 ± 1.7	0.01 ± 0.01
Zn-only	25.5 ± 0.2	7.4 ± 0.05	146.4 ± 2.5	0.01 ± 0.01
Cd-only	25.4 ± 0.1	7.4 ± 0.03	145.5 ± 2.2	0.01 ± 0.01
Cd-Zn	25.5 ± 0.2	7.4 ± 0.03	145.8 ± 2.1	0.01 ± 0.01
Cu-Ni Study				
Control	22.5 ± 0.3	7.6 ± 0.04	149.3 ± 2.7	0.01 ± 0.01
Cu-only	23.3 ± 0.2	7.6 ± 0.05	153.3 ± 2.2	0.01 ± 0.01
Ni-only	23.3 ± 0.2	7.6 ± 0.05	157.3 ± 4.3	0.01 ± 0.01
Cu-Ni	23.1 ± 0.4	7.7 ± 0.06	154.0 ± 1.8	0.01 ± 0.01

Table A2: Larval endpoints of eggs hatched following a 21-day exposure of adult fathead minnows to different experimental treatments. Data are presented as mean ± SEM. All endpoints were assessed using a 2-way ANOVA.

	Control	Cd-only	Zn-Only	Cd-Zn
Hatching Success (%)	70.7 ± 6.4[a]	66. ± 11.0 [a]	71.3 ± 4.9 [a]	72.0 ± 4.5 [a]
% Normal Larvae	92.4 ± 6.4 [a]	94.4 ± 4.0 [a]	96.8 ± 5.0 [a]	86.5 ± 5.1 [a]
Spawn Frequency	0.11 ± 0.02 [a]	0.13 ± 0.04 [a]	0.09 ± 0.02 [a]	0.07 ± 0.02 [a]